Methanogenesis in phytotelmata:

Microbial communities and methane cycling in bromeliad tanks and leaf axils of oil palms

Dissertation

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vorgelegt von

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„Denn neben der objektiven Welt der Materie, die von der Wissenschaft so hervorragend erforscht wird, gibt es die subjektive Welt der Empfindungen, Gefühle, Gedanken sowie der ethischen Werte und spirituellen Hoffnungen, die in ihnen gründen. Wenn wir diesen Bereich außer Acht lassen und ihn so behandeln, als spiele er keine maßgebliche Rolle für unser Verständnis der Wirklichkeit, verzichten wir auf den Reichtum unserer Existenz, und unsere Einsicht wird nicht vollkommen sein. Die Wirklichkeit, einschließlich unseres eigenen Lebens, ist weitaus komplexer als die Beschreibungen des objektiven, wissenschaftlichen Materialismus."

Der 14. Dalai Lama

Die Untersuchungen zur folgenden Arbeit wurden von Januar 2012 bis März 2015 am Max-Planck-Institut für terrestrische Mikrobiologie in Marburg in der Abteilung Biogeochemie unter Anleitung von Prof. Dr. Ralf Conrad durchgeführt.

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Chapter 2
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Chapter 4
Brandt FB, Martinson GO, Pommerenke B, Conrad R. Drying effects on the bacterial community in tank bromeliad slurry incubated inside and outside of bromeliad tanks. **In preparation**.

Content

Summary

The global emission of methane (CH_4) is estimated to be 500-600 Tg per year from diverse natural and man-made sources. Wetlands are the main source of methane and provide an ideal habitat for anaerobic methanogenic archaea which significantly contribute to the total global methane emission. Besides permanently flooded wetlands, there are distinct wetlands created by small water bodies within parts of plants, called phytotelmata. These water catchments in tropical forests comprise bamboo nodes, pitcher plants, tree holes, tank bromeliads and non-bromeliads leaf axils. Recent work indicates that phytotelmata may contribute to the global methane budget. Tank bromeliads, which effectively collect rainwater and organic substrate between their leaf axils (tank slurry), emit substantial amounts of methane into the atmosphere over neotropical forests. However, studies on the microbial communities involved in methane cycling and environmental factors which influence their activity are still rare. In the present study we established tank bromeliads as a model system in the greenhouse and collected field data to investigate the microbial communities in tank bromeliads.

Investigation of Costa Rican tank bromeliads revealed that inhabiting microbial communities (Bacteria, Archaea) differed between individual plants, although the plants belong to the same species and were growing in the same habitat patch. Major determinants for the individual plants microbial community composition were carbon, nitrogen, oxygen concentrations, and the pH of tank slurries. These factors depend on the incoming rainwater, leaf litter or input by higher organisms (e.g. insects, spiders, birds). Therefore, the site where a tank bromeliad develops may play an important role for the inhabiting microbial communities. In summary, our results indicate that every bromeliad tank is a unique island with respect to its resident microbial community. The presence of methanogens and methanotrophs in all tank slurries further indicates the potential for both methane formation and methane oxidation in the bromeliad tanks.

Besides tank slurry properties we have shown that the availability of water shapes the archaeal and bacterial community in tank bromeliads. Increasing drought resulted in a decrease of methane formation and in a shift from a hydrogenotrophic dominated

community (*Methanobacteriales*) to an aceticlastic (*Methanosaetaceae*) dominated methanogenic community. This trend was also observed in the isotopic signature of produced methane and so hydrogenotrophically derived methane dominated under high moisture. Increasing drought resulted in increasing oxygen exposure for the microoorganisms. We found genes for oxygen detoxifying enzymes in genomes of *Methanosaeta* species, indicating that these methanogens are more oxygen tolerant than previously assumed. With increasing drought the relative abundance of the *Burkholderiales*, mainly represented by the genus *Burkholderia*, more than tripled in tank slurry whereas the bacterial diversity decreased. Furthermore, regardless of the water content or the incubation environment (inside or outside of bromeliad tanks) the genus *Burkholderia* was the most abundant group, indicating its tolerance towards changing water levels which frequently occur in tank bromeliads under natural conditions. Upon drought gene copy numbers of *nifH*, a marker gene for nitrogen fixation known to occur in *Burkolderia* spp. as well as *Methanosaeta* spp., increased. Therefore, this work indicates that tank bromeliads inhabiting microbes are not only involved in carbon cycling but also in nitrogen cycling.

We further investigated the potential of methane formation in non-bromeliad leaf axils. The leaf axils of oil palms create catchments similar to the leaf axils of tank bromeliads where organic matter and rainwater accumulate. In incubation experiments we showed that under water-logged oxic or anoxic conditions methane is formed in this organic material, accompanied by increasing gene copy numbers of *mcrA*, a commonly used marker gene for methanogens. Therefore, our results indicate that leaf axils of oil palms seem to be a potential habitat for methanogenesis.

The results of this work give new insights into the microbial communities and methane cycling in plant leaf axils and emphasize the need to better resolve the role of phytotelmata in the cycling of methane to better understand the global methane budget.

Zusammenfassung

Feuchtgebiete repräsentieren die größte Quelle des Treibhausgases Methan (CH_4), das jährlich mit 500-600 Tg in die Atmosphäre der Erde emittiert wird. Einen signifikanten Beitrag leisten dazu anaerobe methanogene Archaeen, die in Feuchtgebieten ein optimales Habitat finden. Neben permanent gefluteten Feuchtgebieten gibt es eine Klasse von „Kleinstgewässern“ die von Pflanzen gebildet werden. Sie werden als Phytotelmen bezeichnet und umfassen in tropischen Wäldern Bambusknoten, Kannenpflanzen, Baumlöcher und Blattachseln von Pflanzen, wie der von Tank-Bromelien. Es wird vermutet, dass diese einzigartigen Feuchtgebiete einen höheren Beitrag zur jährlichen Methanmission beitragen als bisher angenommen.

Es konnte bereits gezeigt werden, dass Tank-Bromelien, die organisches Material wie auch Regenwasser effizient zwischen ihren Blattachseln speichern können (Tanksubstrat), eine erhebliche Menge Methan in die Atmosphäre über neotropischen Regenwäldern emittieren können. Nichtsdestotrotz existieren nur wenige Studien, die sich mit der mikrobiellen Gemeinschaft in Tank-Bromelien befassen und die Faktoren untersuchen, die diese in ihrer Aktivität beeinflussen. Die vorliegende Arbeit umfasst die Aufnahme von Felddaten als auch von Labordaten, wobei letztere durch die Etablierung von Tank-Bromelien als Modellsystem im Gewächshaus gewonnen wurden. Durch Untersuchungen von costa-ricanischen Bromelien konnten wir zeigen, dass die Zusammensetzung der mikrobiellen Gemeinschaft (Bakterien, Archaeen) im Tanksubstrat zwischen einzelnen Bromelien variierte, obwohl diese derselben Spezies angehörten und sich innerhalb des gleichen Habitats entwickelten. Faktoren wie Kohlenstoff-, Stickstoff- und Sauerstoffgehalt als auch pH variierten zwischen den Tanksubstraten und beeinflussten die Zusammensetzung der mikrobiellen Gemeinschaft. Vermutlich sind die genannten Faktoren stark abhängig vom Einfall des Regenwassers und des organischen Substrats, welches hauptsächlich durch pflanzlichen Detritus oder durch höhere Organismen (z.B. Insekten, Spinnen, Vögel) eingebracht wird. Wir nehmen daher an, dass jede Tank-Bromelie ein individuelles Feuchtgebiet in den Baumkronen neotropischer Regenwälder darstellt, abhängig von dem Standort ihrer Entwicklung. Die Detektion methanogener Archaeen, wie auch methanotropher Bakterien in den Tanksubstraten, lässt außerdem

darauf schließen, dass nicht nur das Potential für Methanproduktion sondern auch für Methanoxidation in Tank-Bromelien vorhanden ist.

Desweiteren konnten wir zeigen, dass neben den Eigenschaften des Tanksubstrats, auch die Verfügbarkeit von Wasser eine signifikante Rolle für die mikrobielle Gemeinschaft und die Methanproduktion in Tank-Bromelien spielt. Zunehmende Trockenheit resultierte in einer Abnahme der Methanproduktion und in einer Veränderung der methanogenen Gemeinschaft. Von einer hydrogenotroph-dominierten Gememeinschaft, repräsentiert durch *Methanobacteriales*, erfolgte die Verschiebung zu einer aceticlastischen Gemeinschaft, repräsentiert durch *Methanosaetaceae*. Einhergehend veränderte sich das Isotopensignal des produzierten Methans und bestätigte die Dominanz von hydrogenotroph produziertem Methan unter einer höheren Wasserverfügbarkeit. Durch den sinkenden Wasserpegel im Tanksubstrat wurden die Mikroorganismen zunehmend Sauerstoff ausgesetzt. In Genomen von *Methanosaeta* sp. konnten wir Gene für sauerstoffdetoxifizierende Enzyme finden und vermuten daher, dass diese Methanogenen eine höhere Sauerstoffresistenz besitzen als bisher angenommen.

Auch die bakterielle Gemeinschaft veränderte sich signifikant nach der Veränderung der Wasserverfügbarkeit. Mit zunehmender Trockenheit stieg die relative Abundanz der *Burkholderiales*, hauptsächlich repräsentiert durch die Gattung *Burkholderia,* um mehr als das Dreifache an, während die bakterielle Diversität signifikant abnahm. Unabhängig von der Wasserverfügbarkeit oder des Inkubationsumfeldes (innerhalb oder außerhalb von Bromelien) repräsentierte die Gattung der *Burkholderia* die dominanteste bakterielle Gruppe. Wir nehmen daher an, dass die *Burkholderia* an die natürlich vorkommenden wechselnden Wasserverfügbarkeiten in Tank-Bromelien angepasst sind. Mit Anstieg der Trockenheit wurde zudem ein Anstieg des Markergens für Stickstofffixierung (*nifH*) beobachtet, das auch in *Burkholderia*- und *Methanosaeta*-Arten vorkommt. Die vorliegende Arbeit belegt daher die Präsenz von Mikroorganismen in Tank-Bromelien, die potentiell in den Kohlenstoff- und Stickstoffzyklus involviert sind.

Neben den Blattachseln von Tank-Bromelien untersuchten wir das Potential der Methanbildung in den Blattachseln von Ölpalmen. Auch hier sammelt sich organisches Material zwischen den Blattachseln an und bietet zudem ein Habitat für Moose und andere Epiphyten. In Inkubationsexperimenten unter anoxischen oder gefluteten

oxischen Bedingungen wurde ein Anstieg der Methankonzentration, wie auch des methanogenen Markergens *mcrA*, beobachtet. Diese Beobachtungen lassen darauf schließen, dass Blattachseln von Ölpalmen ein potentielles Habitat für Methanogenese darstellen.

Die Ergebnisse dieser Arbeit geben neue Einblicke in die mikrobielle Gemeinschaft und den Methankreislauf in Blattachseln, und zeigen zudem die Notwendigkeit die Phytotelmen als potentielle Methanquelle weiter zu erforschen, um den globalen Methanhaushalt besser zu verstehen.

1. Introduction

1.1 Methane and methanogenesis

Together with water vapor, carbon dioxide (CO_2), nitrous oxide (N_2O) and ozone (O_3), methane (CH_4) belongs to the primary greenhouse gases in the atmosphere (Hartmann *et al*., 2013) with a 25 times higher global warming potential than CO_2 (Forster *et al*., 2007). The global CH_4 flux to the atmosphere ranges between 500-600 Tg per year and the CH_4 concentration is increasing since pre-industrial times accounting to currently 1770 ppbv (Denman *et al*., 2007; Wang, 2004; Figure 1.1a). In the atmosphere CH_4 has a half life time of about 9 years (Hartman *et al*., 2013). The main removal is due to chemical oxidation with hydroxyl radicals in the troposphere accounting for around 85-90% (~570 Tg y^{-1}) of the global CH_4 sinks (Kirschke *et al*., 2013; Ehhalt and Prather, 2001). A further sink is the consumption of CH_4 by soils which has been estimated by Ehalt and Prather (2001) to be 30 ± 15 Tg CH_4 y^{-1}. This consumption of CH_4 occurs mainly due to the aerobic oxidation by methanotrophic bacteria which can use CH_4 as their sole carbon and energy source (Curry, 2007; Hanson and Hanson, 1996). The anaerobic degradation of CH_4, coupled to sulfate-reduction, is estimated to be 70-300 Tg CH_4 y^{-1} and reduces the atmospheric CH_4 budget by 10-60% (Reeburgh, 2007; Conrad, 2009). In addition, anaerobic CH_4 degradation coupled to nitrate/nitrite reduction may be operative (Deutzmann *et al*., 2014; Hu *et al*., 2014).

The main global sources of CH_4 are shown in Figure 1.1b. More than 70% of atmospheric CH_4 originates from biogenic sources including natural wetlands, termites, ocean, hydrates, ruminant animals and rice agriculture. About 69% of the total CH_4 sources can be attributed to microbial metabolism- mainly due to anaerobic methanogenic archaea (Conrad, 2009). In 1977 Woese and Fox reported that methanogens were only distantly related to typical bacteria (Woese and Fox, 1977) and so they became the first known archaea presently comprising seven orders: *Methanopyrales*, *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales, Methanocellales* (Conrad, 2007) and the recently described order of *Methanomassiliicoccales* (Borre *et al*., 2014). Methanogens are quite diverse in terms of phylogeny and ecology. Their habitats include marine and freshwater sediments, flooded soils, water bodies of plants (phytotelmata),

termites, anerobic digestors, human and animal gastrointestinal tracts, landfills and geothermal systems. The abundance and presence of single types are dependent on the availability of substrates, pH, temperature and salinity (Liu & Whitman, 2008). Methanogens are described as strict anaerobes and their metabolism involves many O_2-sensitive redox centers (Jarrell, 1985). Therefore, it was assumed for years that methanogens are solely restricted to anoxic environments. Nevertheless, it has also been shown that aerated soils are inhabited by methanogens, mainly members of the *Methanocellaceae* and *Methanosarcinaceae* (Angel *et al*., 2011; Peters & Conrad, 1995). Also *Methanobrevibacter* species, living in the hindgut of termites, are able to reduce the oxygen concentration in their immediate surrounding in agar cultures (Leadbetter and Breznak, 1996). Survival in oxic/oxygenic habitats requires combat against oxidative stress. Seedorf et al. (2004) reported that the function of the coenzyme $F_{420}H_2$-oxidase in *Methanobrevibacter arboriphilus* is the detoxification of O_2. The unique set of genes encoding anti-oxidant enzymes in *Methanocellaceae arvoryzae* MRE50, formerly Rice-Cluster I, indicates that methanogens are aerotolerant (Erkel *et al*., 2006). Superoxide reductases, present in some methanogens (Erkel *et al*., 2006), are considered to be the most important oxygen enzymes detoxifying cytoplasmic superoxide anions in anaerobic microorganisms that are strongly exposed to oxygen (Fournier *et al*., 2003; Jenney *et al*., 1999).

However, in most environments archaeal methanogenesis takes place only in highly reduced, anoxic environments as the final step in the anaerobic degradation of organic matter. There, oxygen and other oxidants like nitrate, sulfate or ferric iron are absent. The formation of CH_4 from organic matter is restricted to a cascade of anaerobic degradation processes and requires the action of further microbial groups degrading complex molecules into simpler compounds:

- Hydrolysis of biologyical polymers (e.g. polysachharides) into monomers (e.g. monosaccharides, amino acids) by fermenting bacteria
- Fermentation of these monomers to fatty acids, alcohols, acetate, formate, H_2 and CO_2 by fermenting bacteria
- Conversion of volatile fatty acids and alcohols to acetate, CO_2 and H_2 by a synthrophic or homoacetogenic microflora (Le Mer & Roger, 2001).

Even though methanogens are taxonomically quite diverse, they are limited to a restricted numbers of energy substrates, composed of three major types: H_2/CO_2 (or formate), methyl-group containing compounds (methanol, methylamines, methylthiol) and acetate.

The dominant pathways of CH_4 formation are due to CO_2-dependent (hydrogenotrophic, a) and acetate-dependent methanogenesis (aceticlastic, b).

a) $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$ $\quad \Delta G° = -135$ kJ/mol CH_4

b) $CH_3COOH \rightarrow CH_4 + CO_2$ $\quad \Delta G° = -33$ kJ/mol CH_4

The complex pathway of methanogenesis requires some unique coenzymes, e.g. coenzyme M. The coenzyme M reductase is the key enzyme reducing methyl-CoM to CH_4 in the last step of methanogenesis (Thauer, 1998). This enzyme complex is reported to be unique to methanogens and present in all known methanogens (Thauer, 1998). Therefore, the *mcrA* gene, encoding the α subunit of the methyl-coenzyme M reductase, has become a *functional marker* to track methanogens in the environment and to study their phylogenetic distribution and dynamic processes (Friedrich, 2005; Luton *et al*., 2002).

Although acetoclastic methanogenesis can be performed by only two genera of methanogens (*Methanosarcina* and *Methanosaeta*), the acetotrophically formed CH_4 contributes to two-third of total CH_4 produced (Conrad *et al*., 2010). However, the relative contribution of H_2/CO_2 and acetate to CH_4 formation can vary (Conrad, 1999) and the different pathways of methanogenesis lead to different isotopic compositions of the produced CH_4. Most biological systems favor the lighter ^{12}C and discriminate against the heavier ^{13}C isotope - somehow in a characteristic way. Therefore, the isotope fractionation factor is like a fingerprint to identify reaction pathways and to differentiate between different types of geobiological processes (Hayes, 2001; Conrad and Claus, 2005; Vavilin, 2012). During hydrogenotrophic methanogenesis the isotopically lighter carbon is strongly preferred, whereas the isotope effect is less expressed when the methyl group of acetate is used for CH_4 production (de Graaf *et al*., 1996; Weimer and Zeikus, 1998). Therefore, isotopic signatures can be used to roughly estimate the global

CH_4 budget and the relative contribution of CH_4 sources (Whiticar, 1993). Such data indicate that the global CH_4 budget may not yet be complete and/or sources are not well understood (Potter *et al*., 1996, Lelieveld *et al*., 1998, Wang *et al*., 2004, Kirschke *et al*., 2013). For instance, satellite measurements of global CH_4 abundance indicate that CH_4 emission from tropical forests are higher than previously anticipated (Frankenberg *et al*., 2005, 2006; 2008), whereby known sources can not sufficiently explain these estimations until today (Veldkamp *et al*., 2013).

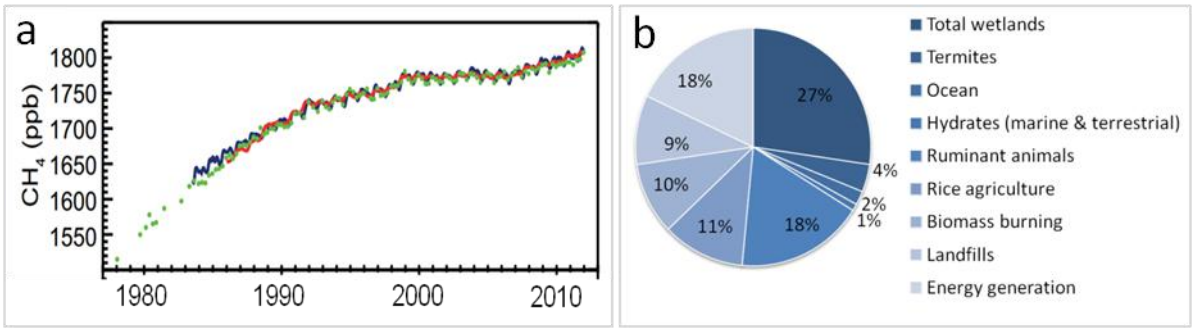

Figure 1.1 a) Globally averaged CH_4 concentration (green; four values per year, except prior to 1984; red; monthly, and blue; quasi-weekly). Figure obtained from IPCC report 2013. b) Methane sources (Lowe, 2006).

1.2 Methane cycling in tropical forests

Tropical forests provide 30% of the terrestrial organic carbon pool (Jobbagy and Jackson, 2000) and have the highest soil-atmosphere CO_2 flux of any biome (Raich and Schlesinger, 1992). Moreover, tropical forests were identified to have a significant influence on the atmospheric concentration of the greenhouse gas CH_4 (Keller *et al*., 1986; Keller and Reiners, 1994; Keller *et al*., 2005). However, conflicting studies report tropical forest soils either as a CH_4 sink (Potter *et al*., 1996) or a source (Silver *et al*., 1999; The *et al*., 2005). This contradiction is likely due to differences in precipitation patterns in tropical forests. Potter et al. (1996) reported that warm and relatively dry tropical ecosystems such as tropical savanna, semi-arid steppe or tropical seasonal forests can represent a major global sink for CH_4. Silver et al. (1999) and The et al. (2005) investigated tropical soils from a site with a high annual precipitation rate (Purto Rico, 3500 to 5000 mm yr^{-1}). There it was shown that tropical soil CH_4 concentrations increased with

increasing annual rainfall, indicating that tropical soil can be an important CH_4 source under these conditions. The amount of rainfall impacts several aspects of soil geochemistry in that increased precipitation may decrease rates of O_2 diffusion into the soil (Keller and Reiners, 1994), resulting in decreased aerobic CH_4 oxidation, and increased anaerobic CH_4 formation. Therefore, precipitation and drought can shift tropical soils from CH_4 sinks to sources and vice versa and therefore are likely to have profound impacts on CH_4 cycling in tropical forests.

Previous studies placing special emphasis on tropical forests as a CH_4 source reported increased annual CH_4 emission (~ 201 Tg) over tropical forests (Frankenberg *et al*., 2006; 2008). One possible explanation for the high CH_4 emission rates was the light-dependent chemical CH_4 production in leaves with annual production rates of up to 236 Tg CH_4 (Keppler *et al*., 2006, 2008). However, the reliability of these estimates was later questioned (Evans, 2007; Beerling *et al*., 2008; Dueck *et al*., 2007). Possible additional sources of CH_4 in tropical forests include termites (Fraser, 1986), poorly drained soil areas (Keller *et al*., 2005), tree stems (Pangala *et al*., 2013), tropical dams (Fearnside and Pueyo, 2012), and CH_4 transport from the soil through the plant transpiration stream (Gauci *et al*., 2010). Furthermore, so-called 'cryptic wetlands', comprising bole depressions and phytotelmata, may significantly contribute to the neotropical carbon cycle. Phytotelmata, water bodies within plants, include the catchments of pitcher plants, bamboo nodes, tree holes, tank bromeliads and non-tank-bromeliad leaf axils (Carmichael *et al*., 2014). Tank bromeliads, belonging to the family of Bromeliaceae, emit substantial amounts of CH_4 into the atmosphere over neotropical forests (Martinson *et al*., 2010). However, the contribution of other phytotelmata to the neotropical CH_4 cycle is still unclear.

1.3 The Bromeliaceae

In 1493 the history of bromeliads began with the second voyage of Christopher Columbus to the 'New World' and the import of the first bromeliad species to Spain: the pineapple (Figure 1.2a). About 200 years later Charles Plumier introduced a group of plants to Europe which he called Bromelia to honor his friend, the Swedish botanist Olaus Bromelius. The family *Bromeliaceae* was divided into the three classic subfamilies of *Bromeliodeae*, *Pitcairnioideae* and *Tillandsiodeae* (Manzanares, 2002). Recently, only the *Bromelioideae* and *Tillandsioideae* are considered to be monophyletic, while *Pitcairnioideae* was further subdivided into five new subfamilies: *Puyoideae*, *Navioideae*, *Hechtioideae*, *Lindmanioideae* and *Brocchinioideae* (Givnish *et al*., 2007). Until today, the *Bromeliaceae* represent one of the largest neotropical families of flowering plants containing about 58 genera with ca. 3140 species (Givnish *et al*., 2011). Bromeliads can grow epiphytically on tree stems or terrestrially on the ground between rocks and mosses (Manzaranes, 2002). The Bromeliaceae are native to tropical and subtropical regions of the neotropics except of *Pitcairnia feliciana* which is, presumable due to recent long-distance dispersal, endemic to western tropical Africa (Figure 1.2b; Smith and Till, 1998). Covering these large areas bromeliads are adapted to many climatic conditions and their habitats range from cloud forests to sun-baked granitic outcrops, to frigid or arid regions (Givnish *et al*., 1997; Benzing, 2000; Manzaranes, 2002).

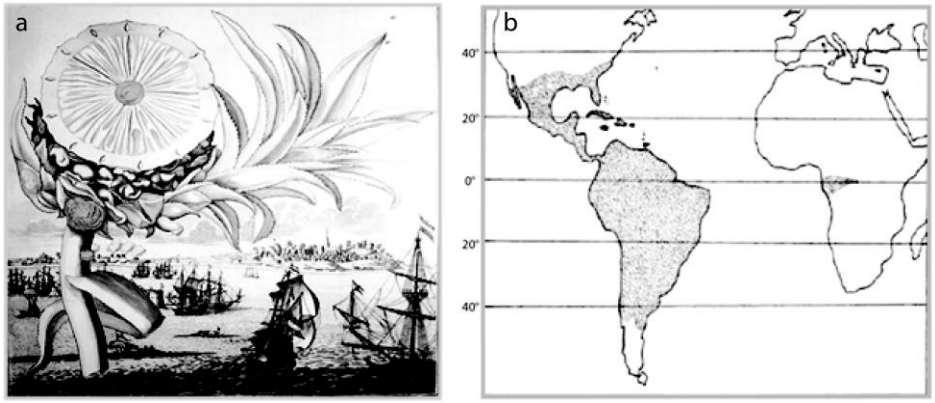

Figure 1.2 a) A pineapple by Johann Christoph Volkamer (1708-1714), b) Distribution of Bromeliaceaea (modified from: Benzing, 2000).

The versatility of the Bromeliaceae is reflected in their photosynthetic lifestyle as well exhibiting the Crassulacean acid metabolism (CAM; ca. 60 %) and/or the C_3 (ca. 30%) pathway (Martin, 1994). Although C_3 bromeliads are often observed in shaded and less stressful habitats and CAM- plants in more arid regions (Martin, 1994) many exceptions exist for both plant types. The CAM photosynthesis allows the plants to avoid stomatal opening during the day when the temperature is relatively high reducing the loss of water by transpiration (Ehleringer and Monson, 1993). Interestingly, there is one species known, which clearly exhibit C_3-CAM alteration. Under sufficient water supply *Guzmania monostachia* exhibits a C_3 photosynthesis. If the plant is exposed to drought, it can switch to CAM (Lüttge, 1986). A further adaption of the *Bromeliacea*e to survive a limited or irregular water supply is the formation of tanks by a densely arrangement of their leaves to store water from rainfalls (Zotz and Thomas, 1999). These bromeliads are often called 'tank bromeliads' and are found in high abundance in neotropical forests (25 - 78 x 10^3 bromeliads ha^{-1}, Sudgen and Robins, 1979; Martinson *et al*., 2010).

1.4 The tank of bromeliads

The creation of tanks by the rosette conformation of overlapping leaves is one of the major morphologic features of bromeliads (Figure 1.3a-d). With these tanks the plant is able to collect and store rainwater and debris (=tank slurry; Figure 1.3e-f) for their nutrient demand (Zotz and Thomas, 1999; Kitching, 2000). Especially epiphytic tank bromeliads are dependent on their tank for their nutrient supply, since their roots are solely used as a holdfast (Zotz *et al*., 2002). Tank bromeliads highly increase the amount of stored carbon and water in the canopies of neotropical forests (Nadkarni, 1994). Contrary to nearby soil, the total organic carbon level in tank bromeliads is often up to ten times higher (35-46 %). The pH is neutral (pH 6.5) to acidic (pH 3.5) and oxygen concentration is low (<1 ppm, Guimaraes-Souza *et al*., 2006; Goffredi *et al*., 2011). This unique ecosystem provide habitats for worms, insects, crabs, snails, frogs, small rodents and microorganisms (Kitching, 2000) and serve as a food source for birds (Silett, 1994). The debris in the tanks, mainly leaf litter, is decomposed by the inhabiting community of organisms which nutrients were incorporated by the plant (Inselsbacher *et al*., 2007). Foliar hairs, so called trichomes, (Figure 1.4), embedded in the leaves of bromeliads,

actively absorb and transport water and dissolved nutrients through the cuticle into the leaves (Benzing and Renfrow, 1974; Pierce *et al*., 2001).

Tank bromeliads are frequently used as natural model systems since their distinct habitats reduce the main problem of ecological studies: the precise delineation of communities (Srivastava *et al*., 2004). Therefore, the plants are used to investigate food web structures (Kitching *et al*., 2001; Srivastava, 2006; Srivastava *et al*., 2008; Brouard *et al*., 2011) animal richness, activity and distribution of (aquatic) invertebrates and microorganisms (Richardson, 1999; Carrias *et al*., 2001; Marino *et al*., 2013). Further, their contribution to the neotropical CH_4 cycle was shown (Martinson *et al*., 2010).

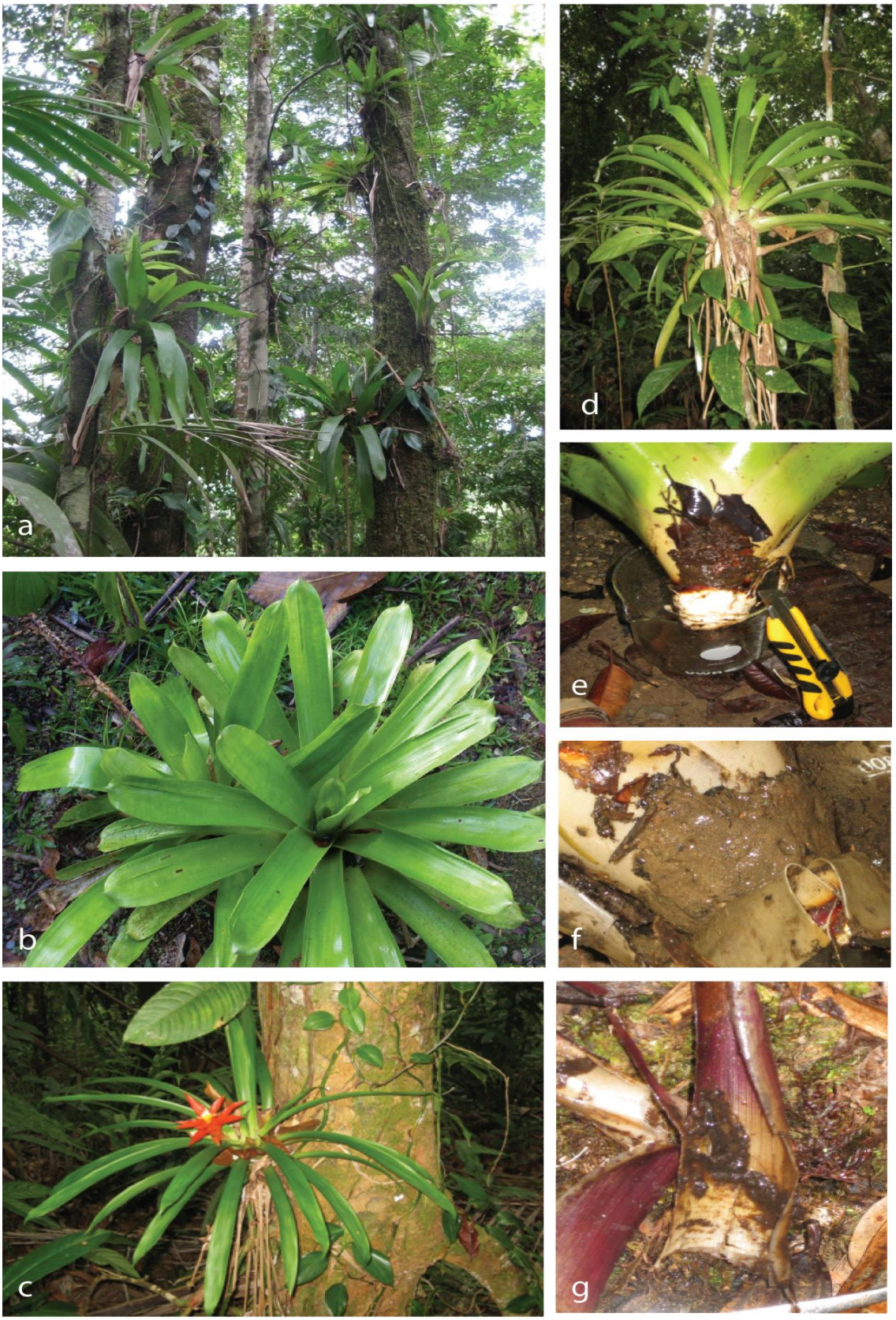

Figure 1.3 Epiphytically growing tank bromeliads in a Costa Rican tropical lowland forest (a). *Werauhia gladioliflora* (b, d) Flowering *Guzmania* (c) Tank slurry located in the leaf axils of *Werauhia gladiliflora* (e, f) from Costa Rica and in *Guzmania* from Ecuador (g).

Figure 1.4 Raster electron microscopic picture (500 x magnification) of bromeliad leaves imaging foliar hairs (trichomes) of a *Guzmania* (a, c) and trichomes and stomata (indicated by arrows) of *Vrisea* (b). Images were taken with the help of *eye of science* (Meckes & Ottawa GbR; scale bars 100 µm).

1.5 Methane cycling in tank bromeliads

The organic matter in tank bromeliads can be anaerobically decomposed by inhabiting microorganism resulting in the release of CH_4. Martinson et al. (2010) first showed that the tank content of bromeliads was supersaturated with CH_4 and potentially influenced carbon cycling when integrating CH_4 release over ecosystem scales. Emissions from tank bromeliads in the canopy of tropical montane forests were estimated to be about 1.2 Tg CH_4 y^{-1}. Molecular analysis showed the presence of common methanogenic phyla in tank bromeliads such as the hydrogenotrophic *Methanomicrobiales, Methanobacteriales, Methanocellales and the aceticlastic Methanosaeataceae* (Martinson *et al*., 2010; Goffredi *et al*., 2011). Also, common soil bacterial phyla were detected in tank bromeliad slurry (*Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Firmicutes*). It has been shown that the bacterial abundance is higher in Costa Rican tank bromeliads than in adjacent soils (Pittl *et al*., 2010). Presumably, these bacteria contribute significantly to the degradation of the tank organic matter (e.g chitin, cellulose) and the production of volatile fatty acids and hydrogen which in turn enables microbial CH_4 formation (Goffredi *et al*., 2011). Nevertheless, little is known about the methanogenic microbial communities in tank bromeliads. For instance, the formation of CH_4 may allow the activity of CH_4-consuming microorganisms (e.g. aerobic methanotrophic bacteria), which has not been studied yet.

Furthermore, understanding the effects of biotic and abiotic factors influencing CH_4 formation is of substantial interest with regard to carbon cycling and accelerated global warming. So far it is known that CH_4 emission from bromeliads correlates with tank diameter (Martinson *et al*., 2010), temperature and nitrogen availability (Kotowska and Werner, 2013). However it can be assumed that further factors including substrate quality and quantity, degree of anoxia and desiccation regulate the formation and release of CH_4. The individual location of a bromeliad may therefore play an important role influencing the receipt of water, leaf litter, nutrients, light radiation and interactions with other organisms. The unique morphology of tank bromeliads allows the plants to effectively collect and store nutrients and water between their leaves. Water saturation in turn enables the creation of anoxic niches for microbial CH_4 formation. However, although the humid tropics are characterized by high annual rainfall, tank bromeliads can face periods of restricted water availability. Evaporation and rainless periods of few hours cause water

stress for the plants, and small individuals can completely dry out (Zotz & Thomas, 1999). Furthermore, the effect of restricted water availability may be increasing in future, since increasing droughts are anticipated in the tropics as a result of anthropogenic climate change and water deficiency in the tank bromeliads may intensify and become more frequent in this century (Cox *et al*., 2008; Malhi *et al*., 2008, Salazar *et al*., 2007). Altogether, these factors may have influence on the CH_4 emission rates and microbial communities in tank bromeliads which have to be taken into account when estimating net fluxes of CH_4 from these phytotelmata.

1.6 Oil palms

The oil palm (*Elaeis guineensis*) is a tropical tree mainly existing in wild, semi-wild or cultivated areas of the equatorial tropics in Africa, South-east Asia and South and Central America (Hartley, 1988). The fruit of the palms is a hard-shelled nut which contains oil. The palms are mainly growing for industrial oil production reaching the highest yield per hectare of all oil crops (Sumathi *et al*., 2008). Oil palm plantations covered 13.2 million ha in 2006 (FAO, 2007) and cultivation is increasing by 9% every year due to expanding biofuel markets (Fitzherbert *et al*., 2008). Further, palm oil is increasingly used for products like margarine, sweets and baked goods, washing powders and cosmetics. The cultivation of oil palms in the tropics often results deforestation of large areas, closely associated with ecological problems and the loss of biodiversity (Fitzherbert *et al*., 2008).

The stem of oil palms is covered by the bases of old leaves which are cutting during crop harvesting or maintenance (Verheye, 2010). Similar to tank bromeliads the leaf axils of oil palms provide a catchment where organic matter accumulates and moss and other epiphytes are growing (Ridley 1930, Figure 1.5). The leaf axils of oil palm presumably provide a unique niche for microbial organisms and a potential place for methanogenesis, since water trapped in leaf axils can lead to development of anoxic conditions and methanogenic colonization (Martinson *et al*., 2010; Carmichaeal *et al*., 2014; Krieger and Kourtev, 2012).

Figure 1.5 Oil palm plantation near Golfito in the south-west of Costa Rica (a). Epiphytes and degraded organic material is located between the leaf axils of oil palms (b, c).

1.7 Objectives of the study

Although small in size, the high number of phytotelmata in tropical forests may result in a significant contribution to the neotropical CH_4 budget. Therefore, it is important to understand and characterize these wetlands as potential habitats for methanogenesis and the inhabiting microbial communities which are potentially involved in CH_4 cycling.

The main objective of this study was to shed light on the methanogenic microbial community colonizing leaf axil environments of tank bromeliads and oil palms. Furthermore, I investigated factors that influence the microbial communities as well as the formation of CH_4. Therefore, one further objective was to establish the tank bromeliad as a model system in the greenhouse.

Chapter 2 | Unique islands - microbial community composition in tank bromeliads of *Werauhia gladioliflora*

Tank bromeliads came into focus as they were identified to emit substantial amounts of CH_4 into the atmosphere over neotropical forests. However, studies on microbial communities which inhabit tank bromeliads are rare. Therefore, the first objective of this study was to investigate the bacterial, archaeal, methanogenic and methanotrophic community in abundance and composition in tanks of mature individual bromeliads. We further determined chemical tank slurry properties (pH, carbon, nitrogen, oxygen concentration, fatty acids) and their explanatory power for microbial community composition. We hypothesized, that the distinct habitat of each bromeliad individual enables the development of plant-specific microbial communities.

Chapter 3| Drying effects on archaeal community composition and methanogenesis in bromeliad tanks

In the previous study we showed that chemical tank slurry properties differed between individual plants and affected the inhabiting microbial communities. These factors may directly depend on the incoming detritus and therefore on the site where a

bromeliad develops. Besides the quality/quantity of incoming organic material, the receipt and storage of rainwater may play a major role for the plants and their inhabiting community. Although the humid tropics are characterized by high annual rainfall, tank bromeliads can face periods of restricted water availability. Furthermore, periods of drought are anticipated to increase in the tropics as a result of anthropogenic climate change and therefore water deficiency for tank bromeliads may intensify and become more frequent. Thus, we investigated if water availability is a controller of the archaeal community composition and CH_4 production in tank bromeliad slurry.

Chapter 4 | Drying effects on the bacterial community in tank bromeliad slurry incubated inside and outside of bromeliad tanks

The bacterial community in tank bromeliad slurry may contribute significantly to the decomposition of organic matter which in turn enables microbial CH_4 formation. Concomitant to the archaeal community characterization, we wanted to determine how the bacterial community in the tank slurry is affected by different water availabilities. A further objective was to check a possible effect of the plant on the bacterial community since most plants are able to influence the microorganisms in their surrounding environment.

Chapter 5 | Leaf axils of oil palms - a potential habitat for methanogenesis

The leaf axils of oil palms create catchments similar to the leaf axils of methane-emitting tank bromeliads where organic matter and rainwater accumulate. We investigated whether the leaf axils of oil palms provide a potential niche for CH_4 formation and a habitat for microorganisms that are involved in CH_4 cycling.

1.8 References

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2. Unique Islands-Microbial community composition in tank bromeliads of *Werauhia gladioliflora*

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Contributions
FBB designed the study, performed the sampling, performed all lab work, evaluated all data, performed statistical analysis and wrote the manuscript
GOM designed the study, performed the sampling, performed statistical analysis and wrote the manuscript
RC wrote the manuscript

2.1 Abstract

Background and aims Tank bromeliads are able to collect organic matter and rainwater (=tank slurry) between their densely arranged leaf axils for their nutrient demand. Diverse communities of microorganisms inhabit these tanks and are responsible for the breakdown of tank organic matter. Anaerobic degradation results in the release of substantial amounts of methane. We hypothesized that each individual bromeliad harbors its own microbial community, the composition being affected by environmental parameters creating unique chemical tank-slurry properties.

Methods We investigated the communities of Bacteria, Archaea, methanogenic and methanotrophic microorganisms measuring their abundance (qPCR) and composition (TRFLP) within eight bromeliad tanks of the species *Werauhia gladioliflora* in a Costa Rican lowland forest. Chemical tank slurry properties were analyzed for pH, carbon, nitrogen, oxygen and fatty acid concentrations.

Results Our results showed that microbial communities differed between plants and were affected by chemical tank slurry properties.

Conclusion Our results indicate that every bromeliad tank is a unique island with respect to its resident microbial community. The presence of methanogens and methanotrophs in all tank slurries further indicates the potential for both methane formation and methane oxidation in the bromeliad tanks.

2.2 Introduction

The Bromeliaceae represent one of the largest neotropical families of flowering plants containing about 58 genera with ca. 3140 species (Givnish *et al*., 2011, Balke *et al*., 2008). The plants grow terrestrially and epiphytically throughout neotropical forest ecosystems. Their densely arranged leaves form tanks that efficiently collect wind-borne particles, leaf litter and rainwater (= tank slurry) for their nutrient demand, and highly increase the amount of stored carbon and water in the canopies of neotropical forests (Nadkarni, 1994). Diverse communities of macro- and microorganisms inhabit these tanks and are responsible for the breakdown of tank organic matter, the release of plant-available nutrients (Ngai and Srivastava, 2006) and the emission of substantial amounts of methane (CH_4) (Martinson *et al*., 2010). Tank bromeliads were used as natural model systems to study food web structures (Kitching *et al*., 2001; Srivastava, 2006; Srivastava *et al*., 2008; Brouard *et al*., 2011), animal richness (Richardson, 1999) activity and distribution of (aquatic) invertebrates (Carrias *et al*., 2001; Marino *et al*., 2013) and microorganisms (Carmo *et al*., 2014). Many different bacteria and archaea, which are commonly found in soils, were also detected in Ecuadorian and Costa Rican tank bromeliad slurries (Goffredi *et al*., 2011a, 2011b; Martinson *et al*., 2010). Due to their high abundance tank bromeliads may therefore represent important habitats for microorganisms involved in the cycling of carbon and nitrogen (Goffredi *et al*., 2011b). Recently, a great variance in the community structure among tank bromeliads were observed by Carmo et al. (2014) investigating the bacterial community in the tank water of *Neoregelia cruenta*. Similar results were reported by Farjalla et al. (2012) evaluating the microbial community in the tank water of four bromeliad species showing that bacteria had no habitat associations as it would be expected from stochastic colonization processes. Environmental controls on microbial community composition in bromeliad tanks have hardly been investigated. Previous studies have shown that the exposure to sun or shade affects the composition and diversity of aquatic insect in the water of bromeliads (Lopez and Rios, 2001). Contrary, Carmo et al. (2014) reported that the bacterial community in tank water showed no grouping by the environmental factors tested (e.g season, sun exposure). The bacterial communities in Costa Rican tank bromeliads were affected by acid-base conditions investigating a range of parameters,

including tank morphology and pH (Goffredi *et al*., 2011b). However, it should be noted that sampling strategies vary between previous studies using either the tank water (Carmo *et al*., 2014; Farjalla *et al*., 2012 Goffredi *et al*., 2011a,b) or the tank debris to investigate the bacterial communities in tank bromeliads (Pittl *et al*., 2010). This may complicate a direct comparison and a restricted sampling may possible lead to an underestimation or exclusion of resident microbial organisms.

Here, we investigated the bacterial, archaeal, methanogenic and methanotrophic communities in the tank slurry of individuals of the species *Werauhia gladioliflora*. We tested the following hypotheses: (1) each bromeliad plant represents a unique island with respect to its microbial community driven by environmental factors which may differ from plant to plant creating unique chemical tank-slurry properties. Environmental effects on the tank slurry of every individual bromeliad may be different and depend on each bromeliads specific growth characteristics and on the environmental conditions of its immediate surrounding (e.g individual architecture and growth position, host tree, chemical composition and quantity of throughfall and light incidence). (2) Environmental effects are not strong enough to cause difference in microbial community composition between leaf axils of a single plant.

In order to reduce potential variability of microbial communities, we tested our hypothesis by selecting eight mature tank bromeliads of the species *Werauhia gladioliflora*, which were of similar size and were grown epiphytically in the same habitat within one hectare of a Costa Rican secondary lowland forest. Since tank bromeliads may contribute to the CH_4 cycle in neotropical forests (Martinson *et al*., 2010) we focused on microorganism that are involved in CH_4 cycling. Thus, we investigated the bacterial and archaeal communities as well as the functional groups of CH_4-producing archaea (methanogens) and CH_4-oxidizing bacteria (methanotrophs) in tank bromeliad slurry. The abundance and composition of the microbial groups were determined targeting the bacterial and archaeal 16S rRNA gene as well as the methanogenic and methanotrophic marker genes *mcrA* and *pmoA*, respectively, using quantitative PCR and TRFLP. We measured the main chemical tank-slurry properties (pH, oxygen, carbon, nitrogen and fatty acid concentrations) and tested their explanatory power for microbial communities.

2.3 Material and methods

Sampling site and chemical analysis

The field station La Gamba, situated in the southwest of Costa Rica on the edge of the National Park Piedras Blancas (N 8°42’61’’, W 83°12'97") encircles an area of 142 km² and is one of the last still virgin rainforests in the lowlands at the Pacific coast in Central America. The climate in this region is dominated by a high rainfall (6,100 mm y^{-1}) (Hofhansl *et al*., 2014). The area around the field station is dominated by tank bromeliads of the genus *Werauhia* (syn. *Vriesea*).

For this study, we collected eight accessible and epiphytically grown tank bromeliads (plant height and diameter ranged between 82-94 cm and 130-140 cm, respectively) of the species *Werauhia gladioliflora* at breast height in an area of approximately 1 ha next to the field station in May 2012. Tank bromeliads were carefully detached from their host trees avoiding loss of water or debris. The tank slurry is defined here as the impoundments of rainwater soaked organic debris located between tank bromeliad leaf axils, which was further used for chemical and molecular characterization.

For comparison between plants tank slurry from three different leaf axils was sampled per plant. For comparison between leaf axils within plants, four out of eight bromeliads were chosen and three additional leaf axils at an opposite position were selected. Dissolved oxygen concentration in the slurry of the central tank was directly measured in the field by a GMH 3630 Digital Oxymeter (Greisinger, Germany). The pH of each tank slurry was measured using a Pocket-Sized pH Meter (Hanna Instruments). Tank slurries were immediately transported in a cooled state (4°C) to the Max-Planck-Institute for Terrestrial Microbiology in Marburg, Germany and then stored frozen at -20°C until analysis. The supernatant of centrifuged tank slurry was membrane-filtered (0.2 µm) and analyzed for fatty acids and ion concentrations using high performance liquid chromatography (HPLC; Sykam) with refractive index and UV detectors and ion chromatography (Sykam), respectively. Fatty acid and ion concentration was calculated using dilution series of external standards (Sigma-Aldrich, Carl Roth). Total carbon and nitrogen concentration of tank slurries were analyzed on a CHNS-elemental analyzer (Analytical Chemical Laboratory, University of Marburg).

Molecular analysis

DNA was extracted from 0.3 g (fresh weight) tank slurry using the NucleoSpin® Soil Kit (Machery-Nagel) following the user manual. The DNA was dissolved in 50 µL nuclease-free water and checked for quality and quantity using a ND1000 spectrophotometer (NanoDrop). DNA was used for all subsequent molecular analyses (qPCR, TRFLP).

Polymerase chain reactions (PCR) for the terminal restriction fragment length polyphormism (TRFLP) analysis were conducted for bacterial and archaeal 16S rRNA genes as well as for the methanogenic marker gene *mcrA,* coding for the methyl coenzyme M reductase subunit A*,* and the methanotrophic marker gene *pmoA*, coding for the methane monooxygenase subunit A. Primer combinations used in the study were: 27f/907r for bacteria (Osborne *et al*., 2005; Muyzer *et al*., 1995) with a 6-carboxyfluorescein (6-FAM) labeled forward primer; 109f/934r for archaea (Grosskopf *et al*., 1998) with 6-FAM-labeled reverse primer; MCRf/MCRr (Springer *et al*., 1995) for methanogens with 6-FAM-labeled forward primer; A189f/mb661r (Costello & Lidstrom, 1999) for methanotrophs with 6-FAM-labeled forward primer. PCR was carried out in a total volume of 50 µL, containing 200 µM desoxynucleotriphosphates (Fermentas), 1x GoFlexiGreen Buffer (Sigma- Aldrich), 10 µg Bovine Serum Albumin (BSA; Roche), 1U GoTaq DNA Polymerase (Sigma-Aldrich), 0.5 µM of each primer pair, 1.5 mM $MgCl_2$ (Promega) and 20 ng template DNA. The PCR reaction was carried out for 94°C for 3 min followed by 22 cycles for bacterial and 30 cycles for archaeal, *mcrA* and *pmoA* gene amplification of 94°C for 30 s, 52 °C for 45 s, 72°C for 90 s and a single step of final elongation of 72°C for 5 min. PCR products were analyzed by 1.5% agarose gelelectrophoresis and visualized by staining with Gel Red™ (Biotium) for 30 minutes. Bands were cut out and purified using Wizard® SV Gel and PCR Clean-Up System (Promega) according to the user manual. Afterwards 100 ng of purified PCR products were digested in a total volume of 10 µL with 2.5 Units of restriction enzyme and the provided reaction buffer with a 1x final concentration. The archaeal 16S rRNA and the *pmoA* amplicons were digested for 6 hours at 36°C using *TaqI* enzyme (Promega), 16S bacterial amplicons were digested at 37°C for 15 min using the *MSPI* Fast Digest® restriction enzyme (Fermentas). The *mcrA* amplicons were digested with *Sau96I* FastDigest® (Fermentas) for 15 min at 36°C. All samples were purified using the SigmaSpin Post-Reaction Clean-up Columns (Sigma-Aldrich) according to the

manufacture's protocol. The size separation was conducted on an ABI PRISM 3130 capillary Genetic Analyzer (Applera Deutschland GmbH) using the software Genescan 4.0 (Applied Biosystems). The TRFLP data were obtained by comparison with an internal DNA standard. The resulting TRFLP profiles were standardized as described in Dunbar *et al*. (2001) using integrated peak area.

The absolute numbers of bacterial and archaeal 16S rRNA, *mcrA* and *pmoA* genes were determined by quantitative PCR (qPCR) using the primer combination Ba519f/Ba907r (Stubner, 2002), Ar364f/Ar934br (Burggraf *et al*., 1997/Grosskopf *et al*., 1998), MCRf/MCRr (Springer *et al*., 1995) and A189F/mb661 (Costello and Lidstrom, 1999). The qPCR was set up in 96-well micro titer plates (BioRad) using a SYBR green I assay. Each qPCR reaction contained in a total volume of 25 µL 1x SYBR®Green Ready Mix™ (Sigma), 3 mM $MgCl_2$ (Sigma), 0.66 µM of each primer and 1 µM FITC (fluorescein isothiocyanat, BioRad) as well as 2 µL of template. Technical replicates were done using 1:10 and 1:100 dilutions of the template DNA. Negative controls without matrix DNA were run in parallel to ensure purity of the used reagents. For all qPCR assays, standards containing known numbers of DNA copies of the respective target gene were used. The quantification standard was applied in a dilution series with $10^1 – 10^7$ gene copies. Thermoprofiles for the quantification of the archaeal 16S rRNA gene copies and *mcrA* gene copies are described by Angel et al. (2011). The quantification of the bacterial 16S rRNA gene was done using the following thermoprofile: initial denaturation 94°C for 8 min, then 45 cycles of 94°C for 20 sec, 50°C for 20 sec and 72°C for 50 sec. The quantification of *pmoA* genes was performed with the following program: initial denaturation step of 94°C for 6 min, then 50 cycles of 94°C for 25 sec, 65.5°C for 20 sec and 72°C for 35 sec for annealing and signal reading. After each quantification a melting curve was performed to ensure the purity of the PCR products.

Statistical analysis

All statistical analyses were conducted in R version 2.10.1. Data of qPCR and chemical properties were tested for normality by Kolmogorov–Smirnov test and for homogeneity of variance by Levene's test and if necessary log-transformed. Means were compared using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Non-metric-multidimensional scaling analysis (NMDS) of microbial community composition was performed based on TRFLP data using Bray-Curtis dissimilarity index. For NMDS analysis we reported a stress value (in %) indicating the lack of the fit between the dimensional mapping of the dissimilarities and the original dissimilarities. Differences between community compositions within a tank bromeliad and among tank bromeliads were investigated using permutational multivariate analysis of variance (Permanova) based on TRFLP results. Microbial community structure was related to environmental factors using canonical correspondence analysis (CCA). Variables which were highly different between tank bromeliads (carbon, nitrogen, oxygen concentration and pH; $P<0.001$; Table 2.1) were included in the CCA model for which significance was tested using ANOVA. The relative contribution of each environmental variable to the total constrained variation was calculated by variation partitioning according to Brocard et al. (1992). Simple linear regression was used to test for a relationship between copy numbers of the bacterial 16S rRNA, archaeal 16S rRNA, *mcrA* and *pmoA* gene to environmental parameters (carbon, nitrogen, oxygen, pH). ANOVA was done using the stats package. NMDS, Permanova and CCA analyses were done using package vegan. All levels of significance were defined at $P<0.05$.

2.4 Results

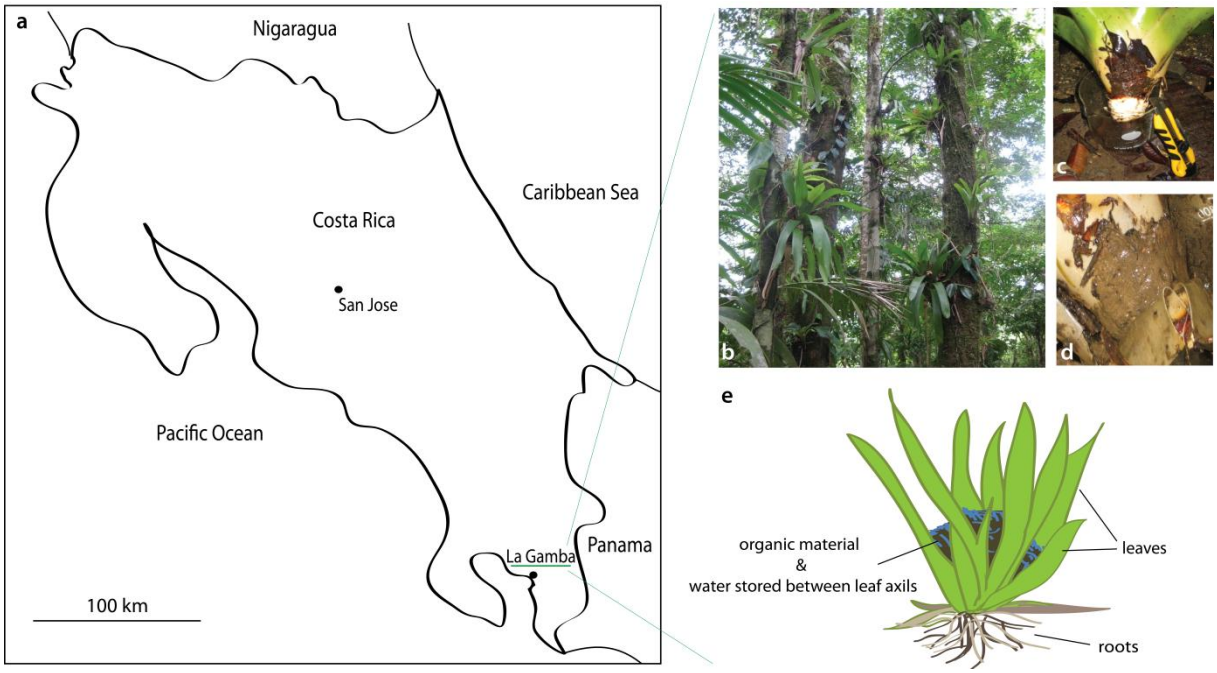

Figure 2.1 Map showing the sampling site selected for this study near the field station La Gamba situated in the Southwest of Costa Rica (a). Epiphytically grown tank bromeliads of the species *Werauhia gladioliflora* (b) were used to sample the tank slurry (organic material and water) between the leaf axils (c, d). Scheme of a tank bromeliad (e).

Tank slurry of *Werauhia gladioliflora*

The tank slurries of all bromeliads collected in a Costa Rican lowland forest (Figure 2.1) showed total carbon and nitrogen concentrations of 39.8 ± 6.9% and 1.7 ± 0.3%, which differed between individual plants (Table 2.1). By comparison, the total carbon content of the adjacent soil was eight times lower (4.9 ± 1.8%) and the total nitrogen concentration six times lower (0.3± 0.1%). The pH of tank slurry and the oxygen concentration of tank water ranged from pH 4.6 - 6.2 and 2.9 - 9.6 mg O_2/L, respectively, and differed between individual tank bromeliads (Table 2.1). Concentrations of lactate, acetate and formate ranged between 0.01 - 1.39 mM, 0.01 - 0.47 mM and 0.05 - 1.15 mM, respectively. Lactate, acetate and formate were found in all tank slurries tested, whereas propionate, heptanoate and butyrate (≤ 0.03 mM) were only detected in up to four tank bromeliads (Table S2.1). Concentrations of fatty acids did not differ significantly between the individual tank bromeliads except formate (Table 2.1). Nitrate was not detectable in any sample.

Table 2.1 Differences in environmental variables among the eight tank bromeliad slurries of *W. gladioliflora* were determined by a one-way analysis of variance (ANOVA).

Variable	df	F	P-value
pH	7	14.0	<0.001*
C_{tot} (%)	7	14.5	<0.001*
N_{tot} (%)	7	14.6	<0.001*
Oxygen (%)	7	17.0	<0.001*
Acetate (mM)	7	2.3	0.08
Lactate (mM)	7	1.4	0.29
Formate (mM)	7	3.6	0.02*

*= significant

Microbial communities in *Werauhia gladioliflora*

Bacterial 16S rRNA gene copies ranged from 3.7 x 10^{10} to 1.8 x 10^{11} copies gdw^{-1} (Figure 2.2). Archaeal 16S rRNA gene copy numbers were one order of magnitude lower than bacterial gene copy numbers. The functional marker gene *mcrA* showed more than 10^9 copies in seven bromeliads (Figure 2.2). The *pmoA* marker gene was determined in five individual tank slurries with more than 10^8 copies (Figure 2.2). Significant lower *pmoA* gene copies (< 5 x 10^6 gene copies gdw^{-1}) were detected in tank bromeliad individuals E and G. The latter one showed significant lower archaeal 16S rRNA gene and *mcrA* gene copies as well (Figure 2.2). Within individual plants no significant differences were observed in bacterial and archaeal 16S rRNA as well as *mcrA* gene copy numbers (Table S2.2). Solely, the *pmoA* gene copy numbers within individual D were significantly different (Table S2.2).

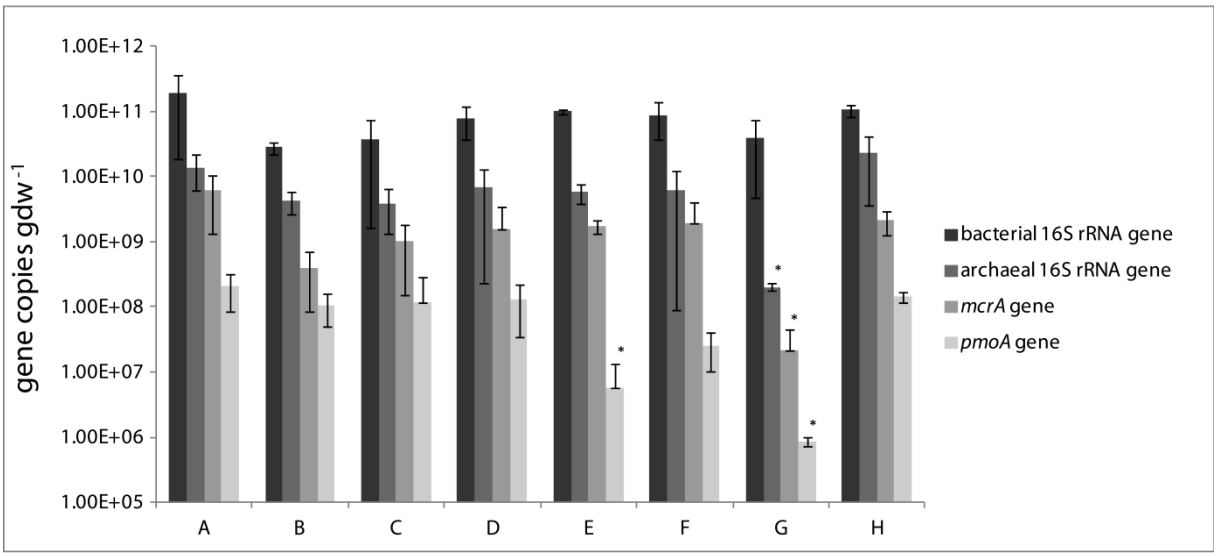

Figure 2.2 Gene copy numbers of bacterial, archaeal, methanogenic and methanotropic communities in the slurry of eight individual tank bromeliads of the species *W. gladioliflora* targeting the bacterial and archaeal 16S rRNA gene and the methanogenic and methanotrophic marker gene *mcrA* and *pmoA*, respectively. Differences in the copy numbers between individual plants (A-H) were determined by a one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test and indicated by an asterisk (*). Error bars represent standard deviation (n=3).

Linear regression was used to test the relationship between copy numbers of the bacterial 16S rRNA, archaeal 16S rRNA, *mcrA* and *pmoA* gene and environmental factors (carbon, nitrogen, oxygen, pH). Solely the number of *pmoA* marker genes correlated significantly with the oxygen concentration (R^2= 0.41, P= 0.03) in the bromeliad tanks. With increasing oxygen concentration the abundance of the methanotrophic community apparently increased in tank slurries of *W. gladioliflora* (Figure 2.3).

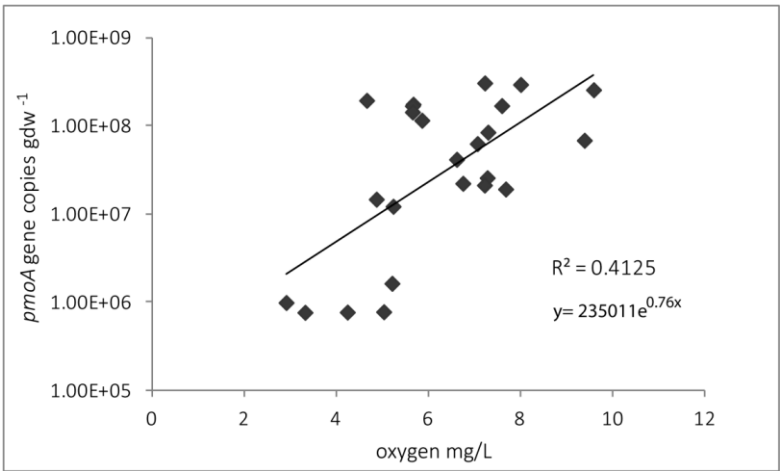

Figure 2.3 Linear regression showing the relationship between oxygen concentration and *pmoA* gene copy numbers in tank slurries of *W. gladioliflora*.

Within individual tank bromeliads no statistical significant differences were observed in the microbial community composition, except for individual C targeting the bacterial 16S rRNA gene (Table S2.2). Among tank bromeliads bacterial, archaeal as well as methanogenic and mathanotrophic community composition differed. In NMDS-plots the bacterial and methanogenic communities displayed a more distinct clustering between individual plants (Figure 2.4a and c) than the archaeal or methanotrophic community (Figure 2.4b and d). This is also indicated by different P-values of the Permanova analysis which were significant for the archaeal (P= 0.004) and methanotropic (P= 0.002) community and highly significant for the bacterial and methanogenic community (P=0.0009). The archaeal and methanotropic community were represented by TRFs which were found in similar abundances in most of the tank bromeliads (Figure S2.1). For instance, the archaeal community was throughout dominated by a 391-bp TRF and in 7 bromeliads the methanotropic community was dominated by a 245-bp TRF representing more than 50% of the relative abundance. Based on NMDS plots representing the archaeal community individual B may be proposed as an outlier. Nevertheless, even after the exclusion of individual B from the analysis the archaeal community composition between the remaining tank bromeliads differed. The NMDS plot of the methanotrophic community may represent individual G as an outlier. After removal of individual G from the analysis the methanotrophic community composition between the remaining tank bromeliads did not differ anymore. Community profiles of all microbial groups in tank bromeliads were different to that in nearby sampled soil (Figure 2.4a-d).

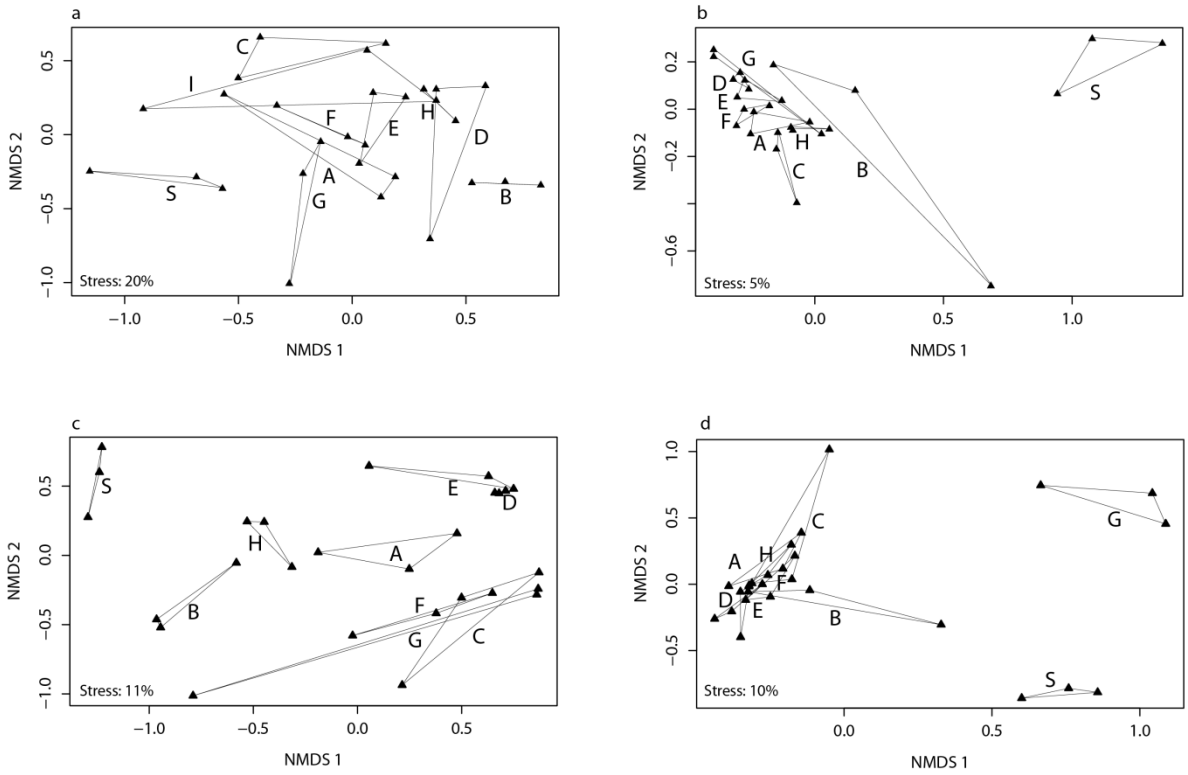

Figure 2.4 Non-metric-multidimensional scaling analysis of the microbial community composition in bromeliad tank substrates of *W. gladioliflora* based on Bray–Curtis dissimilarities using TRFLP profiles of the bacterial 16S rRNA gene (a), archaeal 16S rRNA gene (b), methanogenic marker gene *mcrA* (c) and methanotrophic marker gene *pmoA* (d). Three replicates per plant (A-H) are connected to a triangle and represent the community in a single tank bromeliad and the respective microbial community in nearby sampled soil (S). The closer two points are the more similar they are in community composition. The stress values (in %) indicate the lack of the fit between the dimensional mapping of the dissimilarities and the original dissimilarities.

Canonical correspondence analysis based on TRFLP community profiles were carried out to evaluate the effects of environmental conditions on microbial communities in tank bromeliads. Together, pH, carbon, nitrogen and oxygen concentration affected the bacterial, archaeal, methanogenic and methanotrophic community in tank bromeliads explaining 27%, 28%, 32% and 30% of their variability, respectively (Figure 2.5a-d). Variations in community compositions were significantly explained by the first CCA axis. The first axis sorted the bacterial, archaeal and methanogenic community composition along a gradient from tank substrates with higher C and N and lower pH and oxygen concentrations to tank substrates with lower C and N, and higher pH and oxygen concentrations. Carbon concentration was a fundamental factor controlling the bacterial, archaeal and methanogenic community composition (Table 2.2). For instance, more than

20% of the archaeal community variation was explained by carbon concentration in tank slurry. The methanotrophic community was dominantly sorted by oxygen concentration (Figure 2.5d) which alone explained more than 13% of variation (Table 2.2).

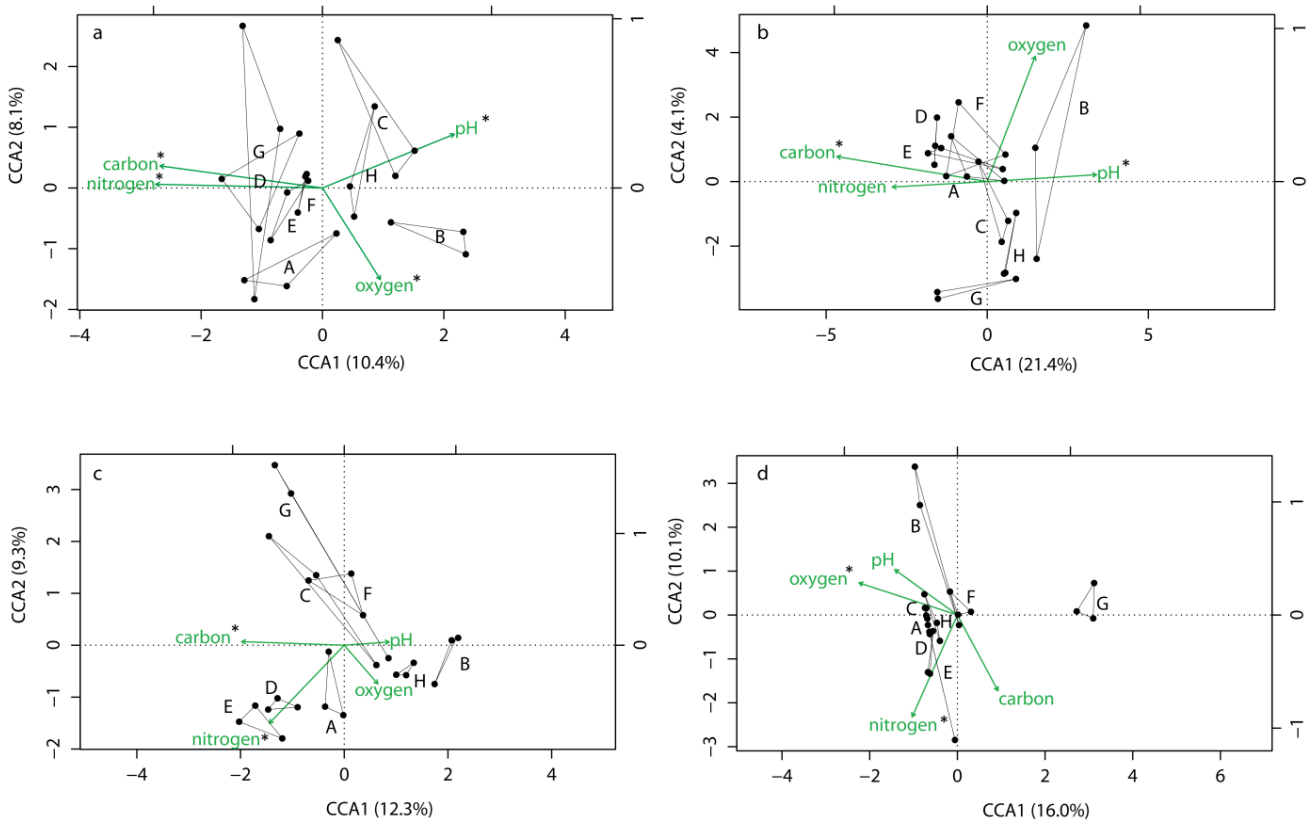

Figure 2.5 Ordination of the bacterial (a), archaeal (b), methanogenic (c) and methanotrophic (d) community across the axis of a canonical correspondence analysis based on TRFLP community profiles. Three replicates per plant (A-H) are connected to a triangle and represent the community in a single tank bromeliad of the species *W. gladioliflora*. The shared effect of carbon, nitrogen, oxygen concentration and pH of tank slurry on the respective community was for each model significant. The significant effect of a single variable is indicated by an asterisk (*).

Table 2.2 Relative contribution of each environmental variable to the total constrained variation for the bacterial, archaeal, methanogenic and methanotrophic microbial community composition in bromeliad tank slurry of *W. gladioliflora*.

Community	Variable	% Explained	P-value
Bacteria	Carbon	10.0	0.005*
	Nitrogen	7.3	0.005*
	pH	8.4	0.01*
	Oxygen	6.6	0.03*
Archaea	Carbon	20.8	0.005*
	Nitrogen	9.7	0.06
	pH	11.8	0.03*
	Oxygen	5.2	0.32
Methanogens	Carbon	11.25	0.05*
	Nitrogen	10.41	0.01*
	pH	6.9	0.15
	Oxygen	5.6	0.20
Methanotrophs	Carbon	7.7	0.11
	Nitrogen	10.9	0.01*
	pH	7.7	0.14
	Oxygen	13.3	0.01*

*=significant

2.5 Discussion

Tank bromeliads came recently into focus as they were identified to emit substantial amounts of CH_4 in neotropical forests (Martinson *et al*., 2010). In this study, copy numbers of the methanogenic marker gene *mcrA* in Costa Rican tank bromeliads showed copy numbers that were similar or even higher than those in a flooded rice field soil (Watanabe *et al*., 2009; Ma *et al*., 2012), which presents a major source of atmospheric methane (Conrad, 2002). Interestingly, methane oxidizing bacteria, quantified by the methanotrophic marker gene *pmoA,* were also found in all eight tank slurries in numbers that were in the same range as observed in wetland sediments or rice field soils (DeJournett *et al*., 2007; Wu *et al*., 2009). Aerobic methanotrophic bacteria are a highly specialized group of microbes using methane as sole source of energy and carbon (Hanson and Hanson, 1996; Murrell and Jetten, 2009) and therefore potentially mitigate methane emission (Frenzel *et al*., 1990). The abundance of the *pmoA* gene was positively correlated with the concentration of oxygen which serves as electron acceptor in methane oxidation and together with methane concentration limits methane oxidation activity. In summary, the presence of methanogens and methanotrophs in all eight individual bromeliads indicates the operation of both methane formation and methane oxidation in bromeliad tanks.

Epiphytic bromeliads increase the volume of arboreal carbon storage by creating catchments in which litter accumulates until it is decomposed (Nadkarni, 1994). Pittl et al. (2010) detected higher bacterial cell numbers in this decomposed litter within tank bromeliads (≥ 10^{10} copies gdw^{-1}) than in tropical canopy soils (taken from the humus pockets generated around tank bromeliad roots) or terrestrial soil, the latter showing the lowest abundance. In the present study, bacterial copy numbers in tank bromeliad slurry were found to be up to one order of magnitude higher (≤ 1.8 x 10^{11} copies gdw^{-1}) than in the study by Pittl et al. (2010). Our results support the assumption that tank bromeliads may provide an ideal habitat for microbial organisms in the canopy of neotropical forests.

Beside differences in community size between tank slurry and and soil samples (Pittl *et al*., 2010), the taxonomic compositions between tank slurries and soil samples differed. These differences may be explainable due to habitat specific conditions. Tank slurries were characterized in this study by a 8 fold higher carbon and 6 fold higher nitrogen content than in the adjacent soil samples and water conditions were previously described to be

different in comparison to nearby freshwater ponds. For instance, some aquatic invertebrates, like the bromeliad ostracod (*Elpidium bromeliarum*) were mainly found in tank bromeliads, which greatly differ in terms of specific composition to nearby ponds and swamps (Little & Hebert, 1996).

Further, our results indicate that each tank bromeliad creates a unique island in the canopies harboring its specific microbial community which mainly do not differ within a plant. It was observed that other plant created water catchments, like tree holes, face episodic turbations of nutrients due to stemflow (Walker *et al*., 1991). Equally, the income of rainwater in bromeliad tanks may lead to turbations of nutrients and inhabiting microbial communities, resulting in a similar community composition within one plant.

However, short generation times of microbes permit rapid changes and adaption (Finlay *et al*., 1997). Therefore, sampling time points or sampling intervals may play a major role in order to detect environmental controls on microbial community composition in tank bromeliad wetlands. In contrast to others, our study captured only a snapshot in time but varied in previous studies up to years (Carrias *et al*., 2001; Goffredi *et al*., 2011b, Carmo *et al*., 2014). However it is to be noted, that due to different water availabilities the microbial community composition in tank slurry can dramatically change even within days (**Chapter 3**, Brandt *et al*., 2014). Here, all tank slurry samples were taken within three days to limit the number of further variations (e.g. seasonal changes, changes in water availability) revealing that the microbial community composition did not differ between leaf axils of a single plant but between individual plants although they belonged to the same species and grew in the same habitat patch. The bacterial and methanogenic communities showed thereby a more distinct clustering between tank slurries than the archaeal or methanotrophic communities did. Especially, the methanotrophic community was dominated by a 245-bp TRF (Figure S2.1d) and did not differ in its composition when one individual was excluded from the statistical analysis. In a previous study this 245-bp TRF was assigned to type-II methantotrophs consisting of various genera of methanotrophic bacteria (e.g. *Methylocystis*, *Methylosinus*; Lüke, 2010). DNA fingerprinting, although a reliable and highly reproducible technique and frequently used for soils and marine environments, shows here a limited phylogenetic resolution. Deeper analysis, using next generation sequencing (e.g. 454 pyrosequencing, Illumina sequencing) would provide higher phylogenetic resolution. Nevertheless, TRFLP analysis for the bacterial, archaeal

and methanogenic community seem to be sufficient enough since significant differences in community compositions were detected between tank slurries. A high bacterial diversity and variability were also observed for other tank bromeliad species (Carmo *et al*., 2014) and habitats within tropical canopies. Differences in bacterial communities within and between tree species were reported by Lambais et al. (2006) investigating the phyllosphere from tree canopies of the Atlantic forests. This pre-existing variability of microbial organisms colonizing the tree phyllosphere may in turn favor the development of unique microbial community compositions in tank bromeliads since incoming leaves serve as nutrient input. Lambais et al. (2006) explained the variations in community compositions due to different leaf ages, location in the canopy, light incidence, and microclimate conditions that influence the leaf environment. These possible explanations go along with our hypothesis that the impact of environmental factors differs between individual plants but may not be strong enough to induce changes in microbial communities between leaf axils of single plants. The individual location of a bromeliad may therefore play an important role and influences the receipt of water, leaf litter, nutrient, light radiation (Guimaraes-Souza *et al*., 2006) and interactions with other organisms (e.g ants; Blüthgen *et al*., 2000). For instance, bromeliads those grow directly under the sunlight receive a higher incidence of light radiation and water but a smaller input of organic matter, contrary to the bromeliads that develop in the shadow of plants (Scarano *et al*., 2002; Guimaraes-Souza *et al*., 2006). Several other studies further indicated that the morphology of a bromeliad species can influence the microclimatic characteristics as well as the community of insects and other macroinvertebrates in the tank of these plants (Jabiol *et al*., 2009, Marino *et al*., 2013). However, the influence of the plant morphology was reduced in this study since we used solely tank bromeliads of the same species and similar size.

To determine chemical factors that influence the microbial community in the tank we investigated tank properties like carbon, nitrogen and fatty acid concentration as well as pH and oxygen concentration which in turn are influenced by the receipt of leaf litter, its quality/quantity and precipitation. Indeed, we clearly observed differences in pH, carbon, nitrogen and oxygen concentration between plant slurries and especially carbon content revealed to be a major driver for the bacterial, archaeal and methanogenic community composition. Carbon availability was already identified as one of the main drivers of

microbial community structure in soil (Fierer *et al*. 2003) and Lopez et al. (2009) assumed that occasional organic matter inputs can induce a eutrophic condition in tank-bromeliads. In a previous study by Goffredi et al. (2011b) it was shown that pH has an effect on the bacterial community composition and a low water content and therefore an increased oxygen exposure, affected the bacterial and archaeal community composition in tank bromeliad slurry (**Chapter 3**, Brandt *et al*., 2014; **Chapter 4**)**.** In the present study, the microbial communities were further sorted along nitrogen concentrations of tank slurry. Nitrogen is a limited factor for tank bromeliads. The plants receive nitrogen by mineralization of organic material from the canopy, from atmospheric sources (Stewart *et al*., 1995), by interactions with animals (Davidson & Epstein, 1989; Leroy *et al*., 2013) or probably by microbial N_2 fixation (Brighigna *et al*., 1992).

Finally, we assume that the place where a tank bromeliad develops seem to be important since carbon and nitrogen concentration of tank slurries are parameters which solely depend on (incoming) detritus as the basal resource and affect the inhabiting microbial communities. Nevertheless, the explained microbial variation due to measured variables in this study was relatively low. On the one hand this can be due to environmental factors that were not measured, like water volume which was shown to affect invertebrate diversity and the archaeal and bacterial community composition and the pathway of methane formation in tank bromeliads (Dézerald *et al*., 2014; **Chapter 3**, Brandt *et al*., 2014; **Chapter 4**). On the other hand there is probably a large stochastic component associated with the ephemerality of the plants and due to dispersal dynamics in the system (Farjalla *et al*., 2012; Jocque and Field, 2014). Tank bromeliads are highly dynamic systems with high emigration rates since many inhabitants of the bromeliads can fly or use vectors that are mobile (Lopez *et al*., 2002).

Conclusion

To our knowledge this is the first study quantifying the *pmoA* marker gene and revealing the presence of methanotrophic bacteria in tank bromeliad slurries, potentially able to oxidize methane. We further showed that tank bromeliads create unique habitats for microbial organisms which do not differ in their composition within a plant but to adjacent soil. Interestingly, differences were also observed between individual plants even belonging to the same species growing in the same habitat patch. Carbon, nitrogen,

oxygen concentration and pH differed between tank slurries and were identified to affect microbial community compositions. We assume that these parameters differ from bromeliad to bromeliad due to local-specific conditions and dependent on the receipt of nutrient inputs, rainwater, or animal interactions. Tank bromeliads are natural microcosms combining the whole ecosystem complexity and increase the microbial diversity and versatility in the canopies of neotropical forests.

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2.7 Supplementary material

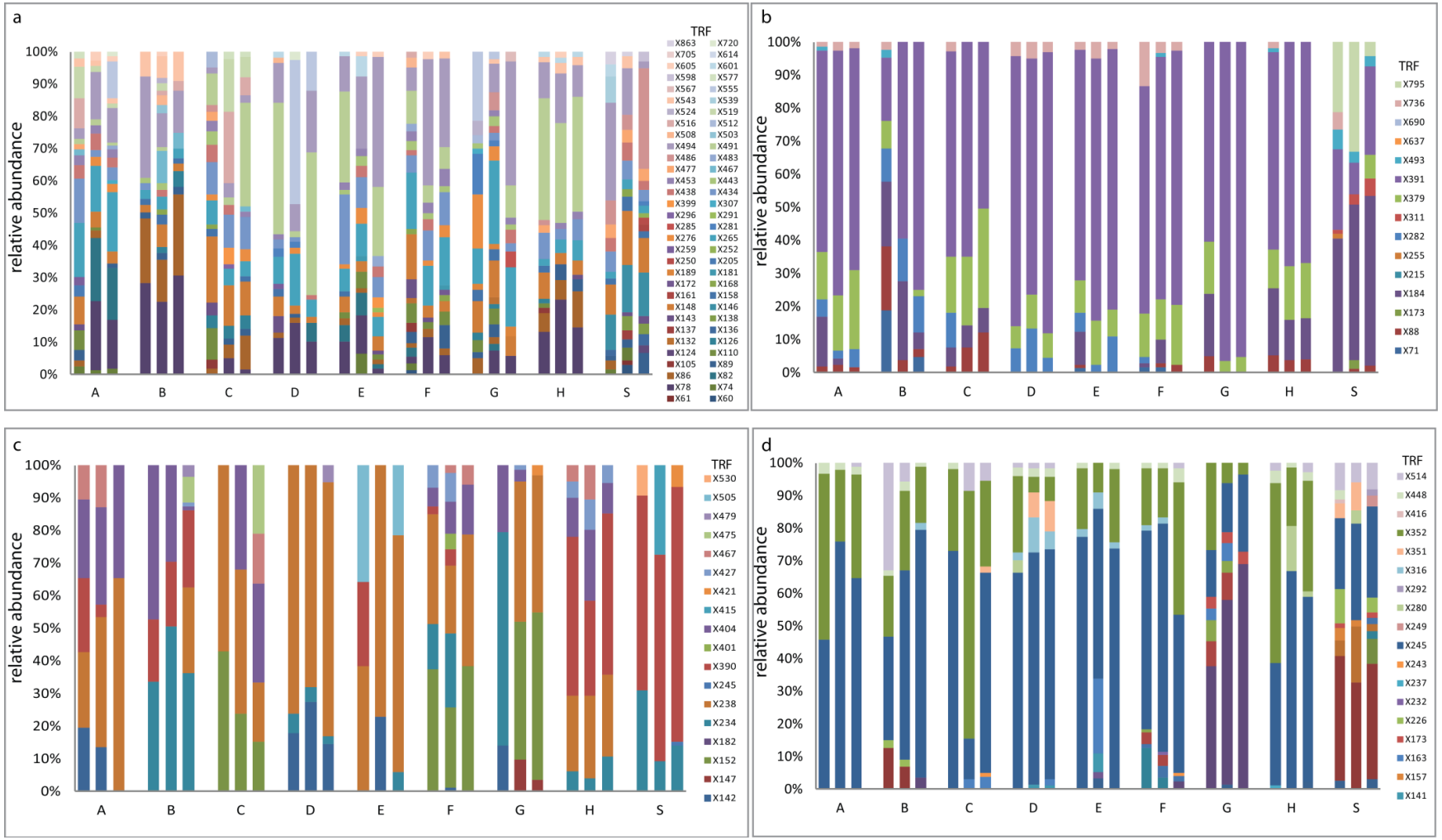

Figure S2.1 TRFLP profile of the bacterial (a), archaeal (b), methanogenic (c) and methanotropic (d) community in tank bromeliad slurry from 8 plants of the species *Werauhia gladioliflora* (A-H) and soil samples (S). The graphs show the relative abundances of TRFs as a measure of the community composition by targeting the bacterial 16S rRNA gene, the archeal 16S rRNA gene and the methanogenic and methanotrophic marker gene *mcrA* and *pmoA*, respectively.

Table S2.1 Tank slurry properties of eight tank bromeliads (A-H) of the species *Werauhia gladioliflora.* All data were obtained from three leaf axils of one individual (1-3).

tank bromeliad	fatty acids						physico-chemical parameters				gene copy numbers			
individual	lactate [mM]	formate [mM]	acetate [mM]	propionate [mM]	heptanoate [mM]	butyrat [mM]	pH	oxygen (mg/L)	N_{tot} (%)	C_{tot} (%)	bacteria	archaea	*mcrA*	*pmoA*
A1	0.08	0.69	0.14	BDL	0.02	BDL	4.81	7.99	2.10	44.29	5.97E+10	9.33E+09	2.54E+09	2.95E+08
A2	0.01	0.39	0.08	0.03	0.01	BDL	4.75	9.38	2.10	46.33	1.25E+11	9.55E+09	4.13E+09	6.88E+07
A3	0.98	0.96	0.24	0.02	0.10	BDL	4.72	9.58	1.68	44.75	3.79E+11	2.26E+10	1.14E+10	2.57E+08
B1	0.03	0.69	0.10	0.00	0.00	BDL	5.54	7.58	1.12	31.12	2.13E+10	4.92E+09	7.41E+07	1.70E+08
B2	0.02	1.15	0.31	0.04	0.01	0.02	5.53	7.28	1.02	20.29	2.87E+10	5.31E+09	7.08E+08	8.50E+07
B3	0.02	0.67	0.14	0.00	0.03	0.00	5.79	7.05	1.33	24.08	3.40E+10	2.44E+09	4.26E+08	6.31E+07
C1	0.01	0.20	0.04	0.01	0.03	BDL	6.24	7.21	1.80	39.82	2.21E+10	6.11E+09	1.34E+09	3.07E+08
C2	0.01	0.13	0.01	BDL	BDL	BDL	6.06	7.67	2.11	36.50	1.17E+10	1.06E+09	3.90E+07	1.93E+07
C3	BDL	BDL	BDL	BDL	BDL	BDL	5.70	7.27	1.98	33.91	7.78E+10	4.51E+09	1.63E+09	2.59E+07
D1	BDL	BDL	0.14	0.01	BDL	0.00	4.60	4.65	1.92	47.76	8.77E+10	3.12E+09	8.13E+08	1.95E+08
D2	1.39	1.03	0.18	0.02	BDL	0.00	4.78	5.66	2.00	47.50	1.15E+11	1.42E+10	3.74E+09	1.76E+08
D3	0.15	0.28	0.07	0.01	BDL	0.02	4.80	7.21	1.93	44.85	3.28E+10	2.83E+09	9.13E+07	2.14E+07
E1	0.01	0.11	0.02	BDL	BDL	0.02	5.71	5.20	2.14	39.90	1.09E+11	7.96E+09	2.16E+09	1.65E+06
E2	0.17	0.11	0.04	BDL	BDL	0.00	4.80	5.02	2.18	43.32	9.55E+10	4.30E+09	1.72E+09	7.86E+05
E3	0.50	0.34	0.36	0.00	BDL	BDL	5.10	4.86	2.15	44.25	9.69E+10	5.25E+09	1.33E+09	1.48E+07
F1	0.09	BDL	BDL	BDL	BDL	0.01	4.87	6.74	1.57	42.89	4.20E+10	2.66E+09	7.74E+07	2.24E+07
F2	BDL	0.14	0.05	0.01	BDL	0.01	4.98	6.60	1.72	40.20	1.41E+11	1.35E+10	4.37E+09	4.19E+07
F3	BDL	0.05	0.02	0.00	BDL	0.00	5.20	5.22	1.33	43.32	8.10E+10	2.78E+09	1.23E+09	1.23E+07
G1	2.93	1.01	0.47	0.01	BDL	BDL	4.70	2.90	1.59	45.30	7.88E+10	1.93E+08	1.82E+07	1.00E+06
G2	0.23	0.69	0.44	0.00	BDL	BDL	4.64	3.31	1.33	44.97	2.23E+10	2.31E+08	1.57E+06	7.74E+05
G3	0.33	0.19	0.07	0.01	BDL	BDL	4.75	4.23	1.35	41.50	1.65E+10	1.82E+08	4.65E+07	7.79E+05
H1	0.01	0.08	0.02	0.02	BDL	BDL	5.50	5.85	1.48	30.10	8.76E+10	1.33E+10	2.93E+09	1.16E+08
H2	0.00	0.13	0.03	0.01	BDL	BDL	5.28	5.64	1.75	35.98	1.32E+11	1.02E+10	2.31E+09	1.69E+08
H3	0.10	0.15	0.07	0.00	BDL	BDL	5.19	5.64	1.70	36.35	9.73E+10	4.46E+10	1.20E+09	1.44E+08

BDL=below detection limit

Table S2.2 Results of PERMANOVA **(1)** and ANOVA **(2)** analysis, based on TRFLP data, respectively qPCR data to detect differences of bacterial, archaeal, methanogenic and methanotropic community composition or abundance in the slurry within individual tank bromeliads of the species *W. gladioliflora* (A-D) targeting the bacterial and archaeal 16S rRNA gene and the methanogenic and methanotrophic marker gene *mcrA* and *pmoA*, respectively.

(1) Community composition				**(2) Community size**			
Plant	**Bacteria**			**Plant**	**Bacteria**		
	df	F	P		df	F	P
A	1	2.13	0.108	**A**	1	1.20	0.33
B	1	3.76	0.113	**B**	1	2.00	0.23
C	1	2.66	0.001*	**C**	1	0.32	0.56
D	1	7.27	0.114	**D**	1	0.38	0.57
	Archaea				**Archaea**		
	df	F	P		df	F	P
A	1	4.93	0.099	**A**	1	0.05	0.84
B	1	0.85	0.406	**B**	1	0.32	0.60
C	1	2.35	0.089	**C**	1	0.18	0.67
D	1	13.21	0.080	**D**	1	0.9	0.39
	Methanogens				**Methanogens**		
	df	F	P		df	F	P
A	1	2.01	0.294	**A**	1	4.29	0.11
B	1	1.32	0.295	**B**	1	4.27	0.11
C	1	2.16	0.404	**C**	1	3.48	0.14
D	1	7.59	0.11	**D**	1	0.89	0.39
	Methanotrophs				**Methanotrophs**		
	df	F	P		df	F	P
A	1	6.96	0.111	**A**	1	0.12	0.744
B	1	1.06	0.301	**B**	1	0.7	0.47
C	1	0.30	0.493	**C**	1	0.6	0.81
D	1	3.95	0.108	**D**	1	15.67	0.017*

*=significant

3. Drying effects on archaeal community composition and methanogenesis in bromeliad tanks

Brandt FB, Martinson GO, Pommerenke B, Pump J, Conrad R (2014) Drying effects on archaeal community composition and methanogenesis in bromeliad tanks. FEMS Microbiology Ecology, fiu021

Contributions

FBB designed the study, cultivated bromeliads in the greenhouse, performed greenhouse-experiment, performed the lab work (probe-specific qPCR, oxygen measurements, gas measurements, isotopic measurements, HPLC and analytical measurements), performed phylogenetic analysis of clone libraries, evaluated all data, performed statistical analysis and wrote the manuscript

BP performed lab work (extracted nucleic acids, qPCR, cloning and T-RFLP)

JP designed the study, supported greenhouse experiment

GOM provided tank bromeliad slurry, designed the study, cultivated bromeliads in the greenhouse, performed greenhouse experiment and wrote the manuscript

RC wrote the manuscript

3.1 Abstract

Tank bromeliads are highly abundant epiphytes in neotropical forests and form a unique canopy wetland ecosystem which is involved in the global methane cycle. Although the tropical climate is characterized by high annual precipitation the plants can face periods of restricted water. Thus, we hypothesized that water is an important controller of the archaeal community composition and the pathway of methane formation in tank bromeliads. Greenhouse experiments were established to investigate the resident and active archaeal community targeting the 16S rDNA and 16S rRNA in the tank slurry of bromeliads at three different moisture levels. Archaeal community composition and abundance were determined using TRFLP and quantitative PCR. Release of methane and its stable carbon isotopic signature were determined in a further incubation experiment under two moisture levels. The relative abundance of aceticlastic *Methanosaetaceae* increased up to 34% and that of hydrogenotrophic *Methanobacteriales* decreased by more than halve with decreasing moisture. Furthermore, at low moisture levels methane production was up to hundredfold lower (≤ 0.1 - 1.1 nmol gdw^{-1} d^{-1}) than under high moisture levels (10-15 nmol gdw^{-1} d^{-1}). The rapid response of the archaeal community indicates that the pathway of methane formation in bromeliad tanks may indeed be strongly susceptible to periods of drought in neotropical forest canopies.

3.2 Introduction

Besides carbon dioxide (CO_2) and water vapor, methane (CH_4) is an important greenhouse gas with a global warming potential 25 times that of CO_2 (Denman *et al*., 2007). Sites of CH_4 formation are generally anoxic soils or sediments (Conrad *et al*., 2002). Therefore, wetlands represent the largest source of CH_4 production by methanogenic archaea (Conrad, 2009). However, known neotropical wetland sources do not sufficiently explain the observed amounts of emitted CH_4 over neotropical forest canopies (Frankenberg *et al*., 2008, doCarmo *et al*., 2006). Tank bromeliads and other cryptic wetlands may contribute to the neotropical CH_4 budget (Martinson *et al*., 2010; Yavit *et al*. 2010). Tank bromeliads are common and highly abundant epiphytes in neotropical forest ecosystems. With their densely arranged leaves they create distinct habitats collecting wind-borne particles, leaf litter and rainwater for their nutrient demand (Zotz & Thomas, 1999). The tanks are habitats for a diverse community of organisms, including microorganisms, insects and invertebrates (Carrias *et al*., 2001; Greeney, 2001; Laessle, 1961) which support a complex food web for the degradation of organic matter until the formation of CH_4 (Martinson *et al*., 2010). In many wetland ecosystems CH_4 formation is due to CO_2-dependent and acetate-dependent methanogenesis. Nevertheless, the relative contribution of H_2/CO_2 and acetate to CH_4 production can vary considerably (Conrad, 1999). The different pathways of methanogenesis result in different isotopic composition of the produced CH_4. The isotopic signatures can be used to constrain the global CH_4 budget and to determine the location and relative contribution of different CH_4 sources (Whiticar, 1993). Although, archaea able for aceticlastic and hydrogenotrophic methanogenesis were detected in tank bromeliads (Martinson *et al*., 2010; Goffredi *et al*., 2011) the relative contribution of these two pathways to CH_4 production is not yet known.

Understanding the effects of biotic and abiotic factors influencing CH_4 formation is of substantial interest with regard to carbon cycling and accelerated global warming. Temperature (Westermann, 1993), plant types (Bartlett *et al*., 1992), primary production (Whiting & Chanton, 1993), CO_2 concentration in the air (Saarnio *et al*., 1998), and water table level (Moore & Knowles, 1989; Frenzel & Karofeld, 2000; Ding *et al*., 2002) were identified to have a significant impact on CH_4 emissions from vegetated wetlands.

Water content could be an important factor for CH_4 emission in tank bromeliads. The unique morphology of tank bromeliads allows the plants to store water between their leaves which in turn creates anoxic niches for microbial CH_4 formation. Although the humid tropics are characterized by high annual rainfall, tank bromeliads can face periods of restricted water availability. Evaporation and rainless periods of few hours may suffice to cause water stress for the plants and small individuals can completely dry out (Zotz & Hietz, 2001). This occurs occasionally since the bromeliads lack an absorptive root system and thus, are entirely dependent on precipitation for water supply (Smith *et al*., 1986). Thus, we hypothesized that water is an important controller of the archaeal community composition and CH_4 production in tank bromeliads. Furthermore, the effect of water availability may be increasing since increasing droughts are anticipated in the tropics as a result of anthropogenic climate change and water deficiency in the tank bromeliads may intensify and become more frequent in this century (Cox *et al*., 2008; Malhi *et al*., 2008, Salazar *et al*., 2007). Greenhouse experiments were established to investigate the resident and active archaeal community by targeting the 16S rRNA gene (16S rDNA) and the reversely transcribed 16S rRNA in composition and abundance in the tank slurry from a plant of the genus *Guzmania*. The incubations were conducted under different water amendments. Simulating a high or low moisture level the tanks of plants were either watered every day (‘wet’) or incubated without any irrigation over one month (‘dry’). Additionally, archaeal community composition in the tank slurry was investigated under drying-rewetting stress by watering the tanks of the plants once per week (‘wet-dry’). In further experiments CH_4 production and stable carbon isotopic signatures of CH_4 formed in the tank slurry were measured under two different moisture levels to determine the methanogenic pathways. Moisture revealed to be a major controller of archaeal community composition and CH_4 production from tank bromeliad slurry.

3.3 Material and methods

Greenhouse experiments

The tank slurry is defined here as a mixture of organic matter and water accumulated in the various leaf axils of a single plant. For our experiments we selected tank slurry from one individual plant of the species *Guzmania squarrosa*, sampled in Ecuador in the south of Loja (adjacent to Podocarpus National Park, detailed site description see Martinson et al. (2013) at 2000 m altitude in May 2011. *Guzmania squarrosa* is the most common bromeliad species at this site. The total tank slurry of the individual plant (348 g) was collected, pooled and immediately transported in cooled state (4°C) to the Max-Planck-Institute for Terrestrial Microbiology in Marburg, Germany, and stored at 4°C. Homogenized slurry (9 g fresh weight) was filled into each central tank of 9 bromeliads of the species *Aechamaea fasciata* 'Primera'. Plants of *Aechmaea fasciata* 'Primera' are commercially available and have the advantage to possess one large central tank even at an early growth stage, thus enabling convenient and reproducible water amendment and sampling. Furthermore, plants of the genus *Aechamea* have frequently been used for greenhouse experiments to study hydrophysiology of the leaves and their acclimation to drought and recovery from dehydration (Londers *et al*., 2005; Ceusters *et al*., 2009). Tank bromeliads of *Aechamaea fasciata* 'Primera' were provided by Corn.Bak® B.V. (Asseldelft, The Netherlands). The plants were 1 year old and of 10 cm size and had been cultivated with empty tanks in the greenhouse.

The experiment was performed in the greenhouse at a temperature of 25 ± 3°C. Different water amendments were conducted on three plants per treatment over 28 days. For treatment 'wet', plants were watered daily by spreading 2 ml deionized water in the central tank where the tank slurry was located. For treatment 'wet-dry' the plants were watered once per week with 2 ml of deionized water. For treatment 'dry', the slurry in the plants was not watered and slowly dried over the period of 28 days.

After 28 days, vertical profiles of O_2 (oxygen) concentrations were measured in the tank slurries of the treatments 'wet' and 'wet-dry' using an oxygen microelectrode (OX50, Unisense, Aarhus, Denmark). In all treatments the pH was measured in the tank slurry.

The water content was determined gravimetrically by drying 1 g aliquot of each tank slurry at 65°C for 72 h.

Subsequently, the tank slurry was sampled for molecular analysis. The leaves were carefully detached and the tank slurry was homogenized. Per plant, 2 aliquots of 0.3 g (fresh weight) tank slurry were taken with a sterile spatula and stored at -80°C till nucleic acid extraction. In total, 6 samples were analyzed per treatment.

Molecular analysis

Nucleic acids were extracted using a modified version of the protocol from Bürgmann et al. (2001). Briefly, the microbial cells in 0.3 g tank slurry were disrupted in a FastPrep®-24 (MP Biomedicals) beat-beating system. Total nucleic acids were subsequently recovered from the supernatant using a phenol/chloroform/isoamyl alcohol extraction (Sigma-Aldrich). The nucleic acids were precipitated with polyethylene glycol (PEG) 6000 solution (Carl Roth) and re-dissolved in 100 µL of nuclease-free water (Invitrogen). The sample was divided and half of the nucleic acids solution (50 µl) was stored at -80°C for later DNA-based molecular analyses. The remaining 50 µL were treated with RNase-free DNase (Qiagen) for removal of DNA in order to obtain pure RNA. The RNA was further purified using the RNeasy Mini Kit (Qiagen). Finally, the RNA was precipitated with 96% Ethanol (Carl Roth), resuspended in 20 µL of nuclease-free water and stored at -80°C. The quality of the RNA was checked using a 1.5% w/v agarose gel (Biozym Scientific GmbH). The RNA concentration was determined using a NanoDrop1000 instrument (Thermo Fisher Scientific). For reverse transcription of RNA into cDNA random hexamer primers (Roche) and SuperScript™ III reverse transcriptase (Invitrogen) were used.

All following molecular approaches were conducted targeting the archaeal 16S rRNA gene (16S rDNA) and reversely transcribed archaeal 16S rRNA to investigate the resident and active archaeal community in tank slurry.

Analysis of terminal restriction fragment length polymorphism (TRFLP) were conducted targeting archaeal 16S rDNA and the 16S rRNA as described by Chin et al. (1999) using the primer combination Ar109f (Grosskopf *et al*., 1998) / Ar912rt-FAM (Lueders & Friedrich, 2003). The reverse primer was labeled with the fluorescent dye 6-carboxyfluorescein (FAM). The purified amplicons were digested using *Taq*I enzyme (restriction site: 5'-

T▼CGA-3`, 65°C; 3 h; Fermentas). The purification of the fragmented DNA was performed using SigmaSpin™ post-reaction clean-up columns (Sigma-Aldrich) following manufactures' manual. The size separation was conducted on an ABI PRISM 3130 capillary Genetic Analyzer (Applera Deutschland GmbH) using the software Genescan 4.0 (Applied Biosystems). The TRFLP data were obtained by comparison with an internal DNA standard. The resulting TRFLP profiles were standardized as described in Dunbar et al. (2001) using the peak area. For comparison see also Martinson et al. (2010).

One clone library based on archaeal 16S rDNA and one based on reversely transcribed archaeal 16S rRNA sequences was generated from pooled triplicates of each treatment ('wet', 'wet-dry', 'dry') using the same primer combination for TRFLP without fluorescent dye. A total of 272 clones were sequenced by GATC Biotech AG (Germany) using the pGEM®-T Easy Vector System (Promega).
All sequences (272) were checked and aligned using ARB program and classified by adding them to the SILVA108 reference tree by parsimony (Pruesse *et al*., 2006). The identity of TRFs was assigned through performing *in silico* enzyme restrictions of clone library sequences. Since the same taxonomic groups were obtained from both approaches (16S rDNA and 16S rRNA) sequences of both libraries were listed in Table 3.1. All sequences that were generated in this study have been deposited in the GenBank nucleotide sequence database (accession numbers KM268122 - KM268393).

The absolute numbers of archaeal 16S rDNA and reversely transcribed 16S rRNA copies were determined by quantitative PCR (qPCR) using the primer combination Ar364f (Burggraf *et al*., 1997) and Ar934br (Grosskopf *et al*., 1998). The qPCR was set up in 96-well micro titer plates (BioRad). Each qPCR reaction contained in a total volume of 25 µl 1x SYBR®Green Ready Mix™ (Sigma), 3 mM $MgCl_2$ (Sigma), 0.66 µM of each primer and 1 µM FITC (fluorescein isothiocyanat, BioRad) as well as 2 µl of template. Negative controls without matrix DNA were run in parallel to ensure purity of the used reagents. The quantification standard was applied in a dilution series with 10^1 – 10^7 gene copies. The standard was prepared from a clone containing the archaeal 16S rDNA as a plasmid insert. The following PCR program was used: 94°C for 8 min followed by 45 cycles of 94 °C

for 20 s, 50 °C for 20 s, 72°C for 50 s for annealing, extension and signal reading. Afterwards melting curves were performed to ensure purity of PCR products.

Dual-labeled probe-specific qPCRs were conducted to quantify the 16S rDNA of *Methanosaetaceae and Methanobacteriales* (Yu *et al*., 2005). Each reaction was conducted in a volume of 25 µl containing the following mixture: 12.5 µl JumpStart *Taq* ReadyMix, 4 mM $MgCl_2$, 0.8 ng μl^{-1} BSA (Ambion), 0.5 µM of each primer, 0.2 µM dual-labeled probe and 5 µl template. The standards for *this assay* were prepared from a clone containing a *16S rDNA* affiliated with *Methanosaetaceae or Methanobacteriales* as a plasmid insert. The following PCR program was used: 94°C for 10 min, followed by 45 cycles of 94 °C for 10 s and 60 °C for 30 s for annealing, extension and signal reading.

Laboratory incubations

Flask experiments were established for determining the methanogenic potential of tank slurry under different moisture contents. The 'wet' treatment had a water content of 28%. Simulating "dry", the tank slurry from *Guzmania squarrosa* (see above) was air dried for one week. The water content had then decreased from 28% to 15%. Each treatment was set up in triplicates with 9 g slurry filled into 125-ml glass flasks that were gas-tightly closed with screw caps including rubber stoppers. The flasks were flushed with synthetic air (21% O_2/ 79% N_2) for 5 min and then incubated in the dark at 25°C for 21 days, until the concentration of CH_4 reached a plateau. Gas samples of 250 µl were taken 2-3 times per week from the headspace using a gas-tight pressure lock syringe (Vici, Baton Rouge, LA, USA) and analyzed immediately. Methane and CO_2 concentrations were analyzed using a gas chromatograph equipped with a methanizer (Ni-catalyst at 350°C, Chrompack, Varian Deutschland GmbH, Darmstadt, Germany) and a flame ionization detector (Shimadzu Deutschland, Duisburg, Germany). Gas production rates were monitored during defined time periods as indicated in Figure S3.1. Oxygen was supplemented 2-3 times per week maintaining atmospheric levels.

Ratios of carbon isotopes ^{13}C:^{12}C in CH_4 and CO_2 were measured using a gas chromatograph combustion isotope ratio mass spectrometer as previously described by

Conrad et al. (2009). The contribution of hydrogenotrophically formed CH_4 to total CH_4 production was calculated after Conrad (2005).

Briefly, the apparent fractionation factor (α_{app}) was calculated by:

$$\alpha_{app} = (\delta_{CO2}+1000)/ (\delta_{CH4}+1000) \quad (a)$$

δ_{CO2} and δ_{CH4} are the isotopic signatures of the carbon in the CO_2 and CH_4. Often α_{app} is expressed as enrichment factor ε_{app} (in ‰) which can be determined through:

$$\varepsilon_{app} = 1-\alpha \quad (b)$$

The relative contribution of the H_2+CO_2-derived CH_4 (f_{H2}) was calculated with the following equation:

$$f_{H2} = (\delta_{CH4}-\delta_{ma})/(\delta_{mc}-\delta_{ma}) \quad (c)$$

where δ_{ma} was determined by:

$$\delta_{ma}= \delta^{13}C_{org} + \varepsilon_{ac,CH4} \quad (d)$$

and δ_{mc} by:

$$\delta_{mc}=\delta_{CO2}+ \varepsilon_{CO2,CH4} \quad (e)$$

δ_{CH4} was the $\delta^{13}C$ of CH_4 measured in the headspace. For calculation of δ_{ma} we assumed $\delta_{acetate}$ to be equal to $\delta^{13}C_{org}$ and $\delta_{ac\text{-}methyl} - \delta_{acetate}$ = -8 ‰ (Conrad *et al*., 2014a). Further, we assumed $\varepsilon_{ac,CH4}$ = -10‰ (Penning *et al*., 2006). Average values for $\delta^{13}C_{org}$ in the slurry were -28‰ (analyzed at the Centre for Stable Isotope Research and Analysis (KOSI) at the University of Göttingen, Germany, courtesy of Jens Dyckmans), thus resulting in δ_{ma} = -46‰. For δ_{mc} a value of -85 ‰ that was typically obtained in incubation experiments, in which aceticlastic methanogenesis was inhibited by 2% methyl fluoride (Conrad *et al*., 2009).

Acetate concentration in tank bromeliad slurry was determined after 21 days of incubation. Prior to measurement, the tank content was mixed with distilled water at a ratio of 1:2, and shaken for 1.5 h at 25°C. The supernatant was sampled with a sterile syringe, membrane-filtered (0.2 µm) and stored frozen until analysis of acetate concentration using high performance liquid chromatography (Sykam, Gilching, Germany) with refractive index and UV detectors (Krumböck & Conrad, 1991). Acetate concentration in the supernatant was determined using dilution series of an external standard solution containing 1 mM acetate (Sigma-Aldrich).

A similar incubation experiment was set up using tank slurry from one adult individual plant of *Guzmania gloriosa* growing at 3000 m altitude. This bromeliad species is very common at this elevation. Treatment 'dry' had a moisture content of 16%, treatment 'wet' of 48%. In this experiment 45 g of slurry was filled into 500 ml serum bottles (n=3 per treatment) and incubated in the dark at 25°C for 15 days, until the concentration of CH_4 reached a plateau. Gas measurements, oxygen supplements, determination of isotopic signatures and methanogenic pathway were conducted as described above. Gas production rates were determined during defined time periods as indicated in Figure S3.2.

Statistical analysis

All statistical analyses were conducted in R version 2.10.1. The effect of moisture on the abundance of archaea, the apparent enrichment factor of ^{13}C in methane and on the methanogenic pathway was assessed using the one-way analysis of variance (ANOVA) and the *post hoc* Tukey (HSD) test. All data were checked for normalty and homoscedasticity using Kolmogorov–Smirnov and Levene's test, respectively. The impact of moisture on the archaeal community based on TRFLP results was analyzed using non-metric multidimensional scaling (NMDS) and *permutational* multivariate analysis of variance (Permanova). All levels of significance were defined at $P \leq 0.05$. For NMDS analysis we reported a stress value (in %) indicating the lack of the fit between the dimensional mapping of the dissimilarities and the original dissimilarities. ANOVA were done using the stats package. Permanova and NMDS analyses were done using package vegan version 2.0-5 (Oksanen *et al*., 2012).

Variation in the distribution of the most dominant TRFs among treatments was assessed using ternary plots representing the relative abundance of each TRF. The ternary diagrams were obtained using the Visualizing Categorical Data package.

3.4 Results

Greenhouse experiment

Gravimetric water content of the tank slurry was at the end of the greenhouse incubation: 29 ± 1% in treatment 'wet', 6 ± 3% in treatment 'wet-dry', and 1 ± 0.5% in treatment 'dry'. Microsensor measurements showed that in the 'wet' treatments O_2 concentrations decreased from atmospheric concentration at the surface of the tank slurry to undetectable concentrations at 2-3 mm depth and below. However, tank slurry of 'wet-dry' treatments still showed an atmospheric O_2 concentration at 3.5 mm depth. The pH values of the tank slurries were slightly higher in 'wet' treatments (pH 5.3) than in 'wet-dry' or 'dry' treatments (pH 4.9). Copy numbers of archaeal 16S rDNA and reversely transcribed 16S rRNA were significantly and oppositely affected by the different water amendments (Figure 3.1). Archaeal 16S rDNA copy numbers were highest in the 'wet' treatment, while copy numbers of archaeal 16S rRNA were lowest in this treatment. A combination of TRFLP fingerprinting with cloning and sequencing was used to identify the archaeal populations under different moisture levels. TRFLP patterns (Figure S3.3) of the archaeal 16S rDNA and 16S rRNA in tank slurry were significantly affected by the different water amendments.

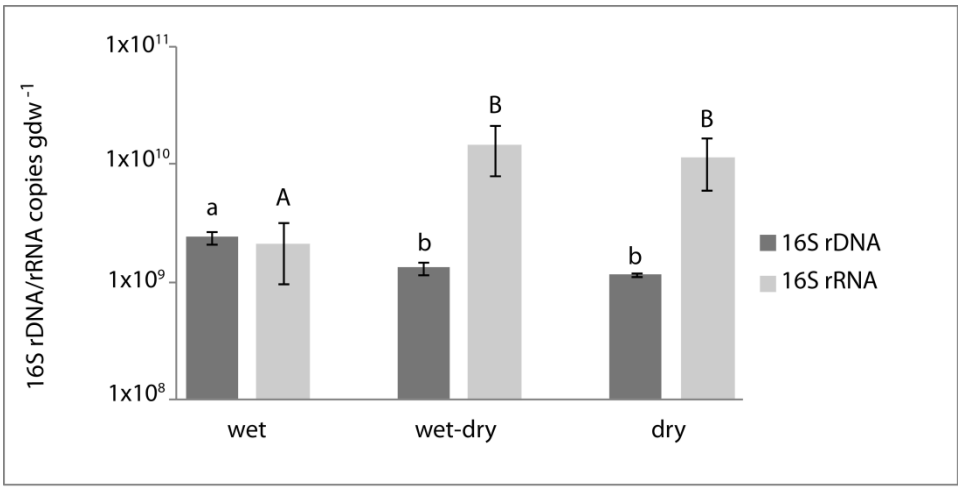

Figure 3.1 Copy numbers of archaeal 16S rDNA and 16S rRNA in tank bromeliad slurry of *G. squarrosa* under different moisture contents after 28 days. Error bars represent standard deviations (n=3). Different letters indicate significant differences between 16 rDNA (lower letters) and 16S rRNA copy numbers (capital letters) (one-way analysis of variance with Tukey's *post hoc* test at $P<0.05$).

The effect of moisture is highlighted in NMDS plots showing the distinct clustering of the resident (Figure 3.2a) and active (Fig. 3.2b) archaeal community according to their treatment. Phylogenetic analysis of clone sequences revealed the presence of *Methanobacteriales*, *Methanosarcinaceae*, *Methanosaetaceae, Methanomicrobiales, Thermoplasmatales* and *Crenarchaeota* in tank bromeliad slurry (Table 3.1). *Methanocellaceae* were only detected as one clone sequence. The major terminal restriction fragments (TRFs) were those with 92, 187, 284, 382, 393 and 736 bp lengths, which were found in all treatments and could be assigned to different archaeal lineages as shown in Table 3.1. Solely the 187-bp TRF was affiliated to more than one lineage (Table 3.1). *Methanocellaceae* were not detected by TRFLP analysis. The microbial community profiles of tank slurry showed that the relative abundance of major TRFs changed with moisture level.

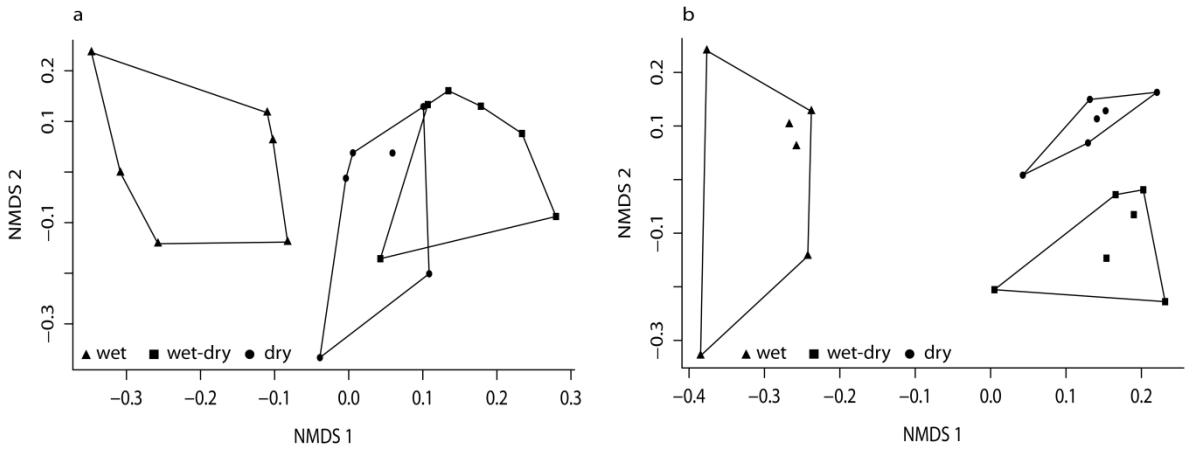

Figure 3.2 NMDS plots showing archaeal communities in tank bromeliad slurry of *G. squarrosa* under different water amendments after 28 days. NMDS plots based on Bray-Curtis distances generated of standardized TRFLP data of the 16S rDNA (a) with stress of 13.7% and 16S rRNA (b) with stress of 11.6%. Different symbols denote the different treatments while samples of the same treatment were graphically grouped.

Table 3.1 Phylogenetic affiliation of distinct terminal restriction fragments to archaeal 16S rDNA/ rRNA clone sequences retrieved from tank bromeliad slurry of *G. squarrosa*.

Phylogenetic lineage	TRF (bp)	No of clones
Methanobacteriales	92	20
Methanosarcinaceae	187	5
Methanocellaceae	259	1
Methanosaetaceae	284	149
Thermoplasmatales	382	18
Methanomicrobiales	393	11
Crenarchaeota	187	13
	736	55
Total		**272**

The relative abundances of single TRFs and affiliated lineages affected by moisture levels are shown in ternary plots (Figure 3.3). Based on 16S rDNA data the most prominent group was *Crenarchaeota* (736-bp TRF), which showed similar signal intensities in tank bromeliad slurry under all water amendments (Figure 3.3a). In contrast, based on 16S rRNA data the 736-bp TRF decreased in relative abundance and was mainly present in slurry of lower moisture (Figure 3.3b). The same phenomenon was observed for the 187-bp TRF, which was affiliated to clones assigned as *Crenarchaeota* and *Methanosarcinaceae*. Further prominent TRFs were presented by the fragments of 92 bp and 284 bp lengths, assigned to the groups of *Methanobacteriales* and *Methanosaetaceae,* respectively. These groups were significantly affected by moisture (Figure 3.4). Based on 16S rDNA data the group of *Methanobacteriales* (92-bp TRF) was dominating with 34 ± 3% relative abundance in the tank slurry of highest moisture. With decreasing water content their relative abundance decreased by more than half (Figure 3.4a). The same trend was observed using 16S rRNA data (Figure 3.4b). In contrast, the abundance of *Methanosaetacea*e (284-bp TRF) increased with decreasing moisture (Figure 3.4c and d). Based on 16S rRNA data the relative abundance of *Methanosaetaceae* doubled from 9 ± 5% in treatment 'wet' to 23 ± 4% in treatment 'wet-dry' and tripled to 34 ± 4% in treatment 'dry' (Figure 3.4d). The increasing dominance of

Methanosaetaceae with decreasing gravimetric water content was also seen as absolute abundance increased, as shown by using a probe-specific qPCR assay. The 16S rDNA copy numbers of *Methanosaetaceae* doubled from $1.5\times10^5 \pm 2.5\times10^4$ copies gdw^{-1} in the 'wet' samples to $2.9\times10^5 \pm 5.3\times10^4$ copies gdw^{-1} in the 'wet-dry' and tripled to $4.8\times10^5 \pm 1.2\times10^5$ copies gdw^{-1} in the 'dry' samples. Contrary, the 16S rDNA copy numbers of *Methanobacteriales* were higher in the 'wet' samples ($6.2\times10^5 \pm 1.1\times10^4$ copies gdw^{-1}) than in the 'wet-dry'($3.9\times10^4 \pm 1.2\times10^4$ copies gdw^{-1}) or 'dry' samples ($1.2\times10^5 \pm 1.9\times10^4$ copies gdw^{-1}.

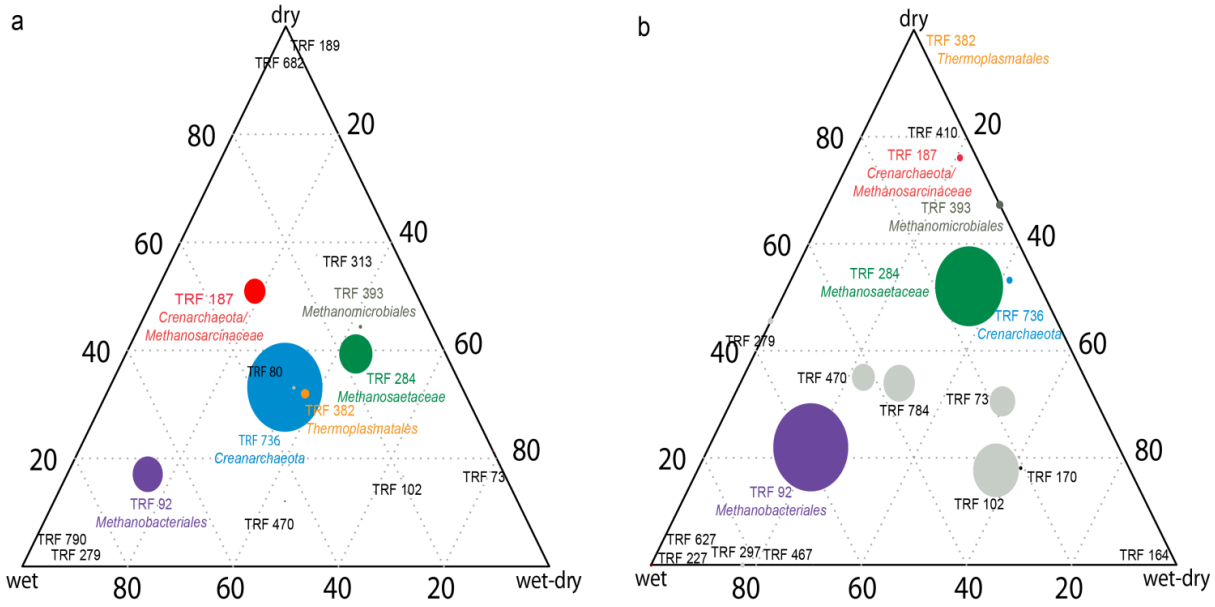

Figure 3.3 Ternary plots of TRFLP data based on archaeal 16S rDNA (a) and 16S rRNA (b) in the tank slurry of *G. squarrosa*. Each circle represents one TRF. The size of each circle represents its relative abundance. Each corner of the triangle represents a proportion of 100% for the respective treatment ('wet',' wet-dry' or 'dry') with the other corners representing 0% of that treatment. As the relative abundance of a TRF in a treatment increases then it moves towards the corner representing that treatment.

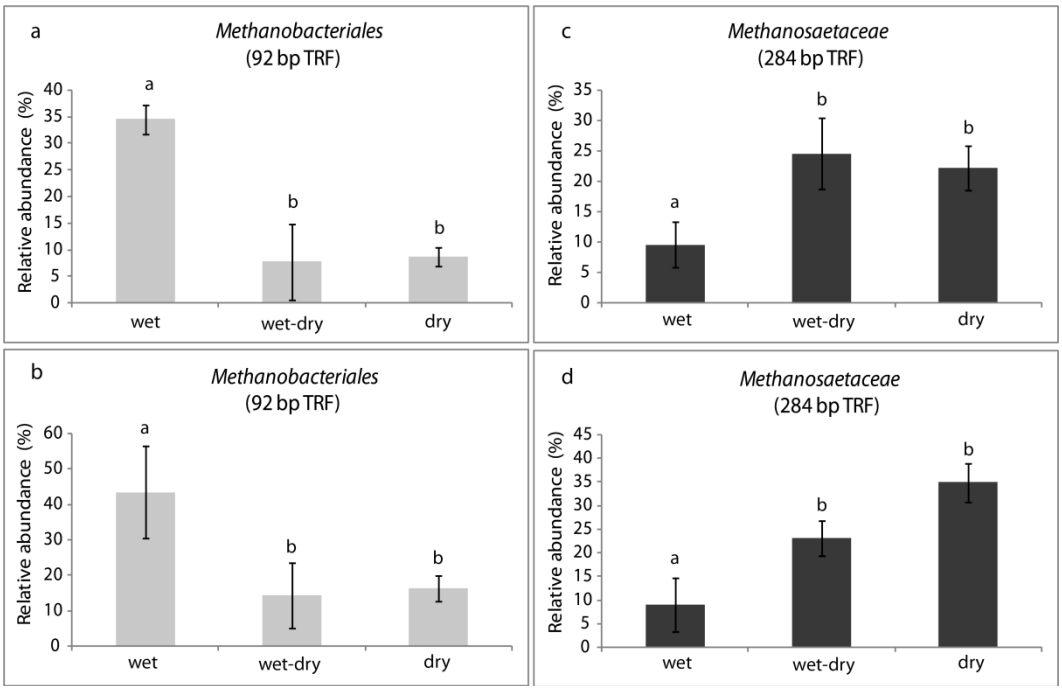

Figure 3.4 Relative abundance of single 16S archaeal TRFs under different water amendments in the tank slurry of *G. squarrosa*. Plots (a) and (c) represent the 92-bp TRF (*Methanobacteriales*) and the 284-bp TRF (*Methanosaetaceae*) based on archaeal 16S rDNA, respectively. Plots (b) and (d) represent the relative abundance of the 92-bp-TRF and 284-bp TRF based on the archaeal 16S rRNA. Error bars represent standard deviations (n=6). Different letters indicate significant differences (one-way analysis of variance with Tukey's *post hoc* test at $P<0.05$).

Laboratory experiments

The CH_4 production rate in tank slurry (sampled from *G. squarrosa*) was determined at two different water contents. After three weeks of incubation, the tank slurries with high moisture content showed higher acetate concentrations (64 ± 32 µM; n= 3) than those with low moisture contents (32 ± 6 µM; n= 3). Gas concentrations of CH_4, CO_2 and O_2 in the headspace of incubation flasks are shown in Figure S3.1. Methane production rates ranged between 10 and 15 nmol CH_4 gdw^{-1} d^{-1} and 0.05 and 1.1 nmol CH_4 gdw^{-1} d^{-1} in the tank slurries at high (28%) and low (15%) moisture content, respectively (Figure 3.5a). The $\delta^{13}C$ values of newly produced CH_4 were between -71 and -73‰ at high and between -50 and -66‰ at low moisture content. Rates of CO_2 production were higher at low (4.3-4.6 µmol gdw^{-1} d^{-1}) than at high moisture (2.1-2.5 µmol gdw^{-1} d^{-1}).

Concentrations of CH_4, CO_2 and O_2 in the headspace of incubation flasks with tank slurry from *G. gloriosa* were determined over 15 days (Figure S3.2). Methane production rates were 11 nmol CH_4 gdw^{-1} d^{-1} to 13 nmol CH_4 gdw^{-1} d^{-1} at high and ≤ 0.1 nmol CH_4 gdw^{-1} d^{-1} at low moisture content (Figure 3.5b). The $\delta^{13}C$ values for CH_4 ranged from -80 to-81‰ at 48% water content and from -56 to -63‰ at 16% water content. Release rates of CO_2 showed similar results as for *G. squarrosa* with 4.3-5.2 µmol gdw^{-1} d^{-1} at low moisture and 1.5-1.7 µmol gdw^{-1} d^{-1} at high moisture. The $\delta^{13}C$ values of CO_2 in the headspace were similar under all treatments and amounted to -28.5 and -29.5‰ to -29.6 and -29.7‰ for the tank slurries of *G. squarrosa* and *G. gloriosa*, respectively.

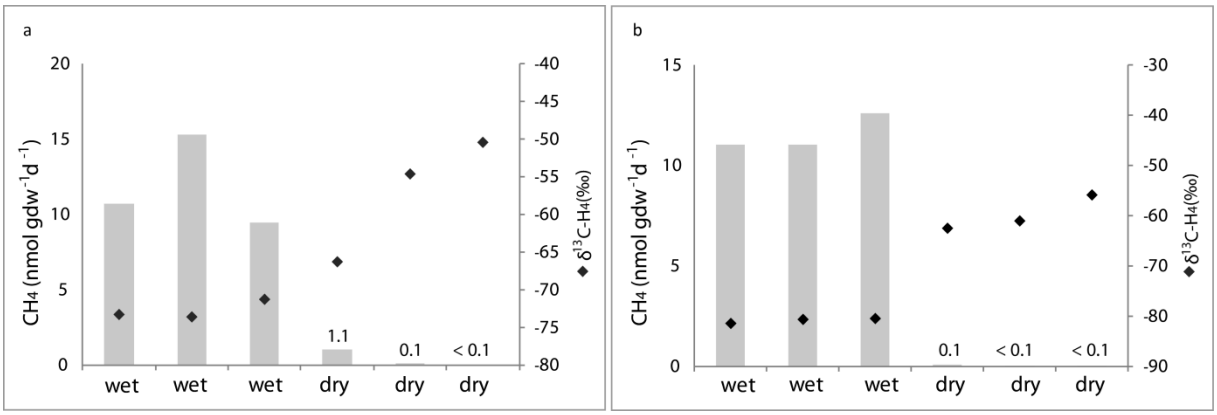

Figure 3.5 Methane production rate of tank bromeliad slurry of *G. squarrosa* (a) and *G. gloriosa* (b) under different water amendments (left axis). Stable carbon isotope signature ($\delta^{13}C$) of the newly formed methane in the headspaces was measured after 21 days for (a) and 15 days for (b) (right axis).

The isotopic signatures of CO_2 and CH_4 were used to estimate the apparent enrichment factors ε_{app} for the two different tank slurries at high and low moisture level (Table 3.2). The values were consistently lower under wet than under dry conditions indicating that the relative contribution of the methanogenic pathways was different (Table 3.2). Indeed, the relative fraction of hydrogenotrophically derived CH_4 was significantly higher in wet ($f_{H2} \geq 0.68$) than in dry tank slurry ($f_{H2} \leq 0.35$).

Table 3.2 Effect of moisture on the apparent enrichment factor of ^{13}C in methane and the methanogenic pathway in tank slurry from bromeliad species of the genus *Guzmania* in wet and dry simulated experiments.

Tank slurry	Treatment	ε_{app}	f_{H2}
G. squarrosa	wet	-48.6 ± 1.9[a]	0.68 ± 0.03[a]
	dry	-29.9 ± 9.0[b]	0.28 ± 0.21[b]
G. gloriosa	wet	-56.6 ± 0.6[a]	0.89 ± 0.01[a]
	dry	-32.3 ± 3.5[b]	0.35 ± 0.08[b]

ε_{app} = apparent enrichment factor (in ‰), f_{H2} = relative contribution of hydrogenotrophically derived methane, ± standard deviation (n=3). Different letters denote significant differences (one-way analysis of variance with Tukey's *post hoc* test at P<0.05)

3.5 Discussion

Moisture was identified to be a major determinant of the archaeal community composition in tank bromeliad slurry of *Guzmania*. For soil ecosystems it has already been shown that moisture can be a dominant driver of bacterial community composition (Evans *et al*., 2014) and that it is important for microbial functioning and activity (Evans & Wallenstein, 2012; Schimel *et al*., 1999; Skopp *et al*., 1990). Especially for tank bromeliads the storage of rainwater is a key parameter to ensure their water supply above the ground. The tanks are inhabited by many aquatic and terrestrial organisms, and water volume in tank bromeliads has already been identified to shape the diversity of inhabiting insects (Armbruster *et al*., 2002). Our results clearly show that the archaeal community composition and size in tank bromeliad slurry changed upon drying. Interestingly, archaeal communities in rice fields as man-made wetland ecosystems are often described as relatively resistant against dramatic changes like drainage or crop rotation (Fernandez Scavino *et al*., 2013; Ma *et al*., 2012; Watanabe *et al*., 2006; Krüger *et al*., 2005; Lueders & Friedrich, 2000). Stress resistance of microbial communities can be influenced by various key factors including microbial richness and evenness (Wittebolle *et al*., 2009) as well as the historical soil moisture regime which may affect the magnitude and timing of microbial community response to drying and rewetting events (Evans & Wallenstein, 2012). Accordingly, an increased resistance to drought was observed for microbial communities in grassland (de Vries *et al*., 2012) when the soil was previously exposed to drying-rewetting events and a poor adaption to drying-rewetting events when the soil lacked a history of drought (Thion & Prosser, 2014). Although tank bromeliads and *Guzmania spp.* in general are very efficient in maintaining moisture in their tanks (Zotz & Thomas, 1999) the plants can be exposed to periods of droughts. However, the levels of relative air humidity at our study sites at 2000 m and 3000 m elevation remain fairly high over the whole year with daily minima of 15 to 30% (Moser *et al*., 2007) and dry conditions become only significant for short periods during intensive summers (Peters *et al*., 2013). Therefore, our drying experiments presently do not represent normal in-situ conditions but the potential under extreme conditions. Likewise, drying of Amazonian lake sediments resulted in changes of archaeal community composition (Conrad *et al*., 2014b). However, the high organic matter content in tank slurry (38 - 45%) may enhance the effect of drying on the archaeal tank community. Orwin and Wardle (2005) reported

that soils rich in organic matter showed a reduced microbial resistance to drying. Soils with high C content have higher water retention and therefore microbial communities may be more protected from changes in moisture contents and less well adapted to drying (Rawls *et al*., 2003).

Forest humidity is decreasing since 1998 (Wilcke *et al*., 2013) and rainless periods of two or more weeks become more frequently at our study site (Peters *et al*., 2013). These changes are in line with climate change effects that are projected to lead to larger periods of drought in the neotropics (Hulme & Viner, 1998; Oliveira *et al*., 2005). These drought-stressed periods in turn may affect rates of organic matter mineralization and production of CH_4 and CO_2 from bromeliad tanks. Here, CO_2 release rates into the headspace of incubation flasks were 2-3 times higher from tank slurry of low than of high moisture, indicating aerobic degradation of organic matter which is postulated to be faster than anaerobic degradation (Reddy & Patrick, 1975). Methane production rates were up to 100 times lower in tank slurry with relatively low moisture content. Additionally, the methanogenic pathway and the archaeal community composition were affected by drought. Our results showed that extended periods of drought resulted in a decrease of the relative abundance of hydrogenotrophic *Methanobacteriales* in tank bromeliad slurry, and in an increase of the aceticlastic *Methanosaetaceae* (Figure 3.4). Consistently, analysis of stable carbon isotopic signatures indicated a decrease of hydrogenotrophic methanogenesis and an increase of aceticlastic methanogenesis with decreasing moisture of the tank slurry.

Our experiments showed that CH_4 was mainly produced by hydrogenotrophic methanogens in tank slurry at high moisture content. Relatively high contribution of hydrogenotrophic methanogenesis is characteristic for lake sediments (Conrad *et al*., 2014b; Namsaraev *et al*., 1995; Galchenko, 1994; Crill & Martens 1983) and for the methanogenic degradation of aged organic carbon (Nakagawa *et al*., 2002). Furthermore, hydrogenotrophic methanogenesis has been found in environments in which acetate is assimilated rather than dissimilated such as in animal gut systems (Liu & Whitman, 2008; Lange *et al*., 2005) or microbial mats (Sandbeck & Ward, 1981).

Our experiments further showed that the contribution of aceticlastic methanogenesis became more important, when the tank slurry became dry. Acetate concentrations at the end of the incubation experiment were also lower in the dry tank slurry. A slight increase

of aceticlastic methanogenesis was also observed by Conrad et al. (2014b) in lake sediments and by Knorr et al. (2008) in a fen soil upon experimental drought. However, lower water tables or drought in our experiment simultaneously implied an increased oxygen exposure to microbial organisms. Methanogenic archaea are described as strict anaerobes (Zinder, 1993) and their metabolism involves many O_2-sensitive redox centers (Zehnder, 1978). Nevertheless, it has also been shown that aerated soils are inhabited by active methanogens (Angel *et al*., 2011; Peters & Conrad, 1995), mainly members of the *Methanocellaceae* and *Methanosarcinaceae* (Angel *et al*., 2011). Subsidiary, *Methanosarcinaceae* was solely detected in tank slurry of low moisture based on 16S rRNA analysis (Figure 3.3b). For *Methanocellaceae* and *Methanosarcinaceae* it is known that they possess a large diversity of genes encoding for oxygen detoxifying enzymes (Angel *et al*., 2011; Erkel *et al*., 2006). Some of these enzymes (catalase, superoxide dismutase, rubrerythrin, peroxiredoxin) were also found in the genomes of *Methanosaeta concilii* (NCBI Reference Sequence: NC_015416.1; Barber *et al*., 2011) and in *Methanosaeta harundinaceae* (NCBI Reference Sequence: CP003117; Zhu *et al*., 2012). We therefore assume that the increase of *Methanosaetaceae* in the dry and therefore aerated tank slurry indicates that *Methanosaetaceae* are also more tolerant to O_2 than previously assumed.

Beside methanogenic archaea, *Crenarchaeota* were dominantly present in tank bromeliad slurry based on 16S rDNA analysis. However, targeting the 16S rRNA and so the more active members of the archaeal community *Crenarchaeota* play a minor role in contrast to the methanogenic community (e.g. *Methanobacteriales*, *Methanosaetaceae*). Members of *Crenarchaeota* have previously been detected in relatively high numbers in different soils (Timonen & Bomberg, 2009). Nevertheless, we do not know which function *Crenarchaeota* may have there and particularly in tank slurry.

In conclusion, our study provides evidence that moisture in the tank slurry is a dominant driver of the archaeal community composition, the archaeal abundance as well as the methanogenic pathway. With increasing drought the methanogenic community shifted from a hydrogenotrophic dominated community, represented by *Methanobacteriales*, to an aceticlastic dominated community, represented by *Methanosaetaceae.* This shift was accompanied by an increase of aceticlastically produced CH_4. Hence, we suggest that

microbial methane cycling in bromeliad wetlands is strongly susceptible to periods of drought in neotropical forest canopies. In order to confirm our assumption, it will be necessary to investigate microbial CH_4 cycling in bromeliad tanks of other bromeliad species and at different sites with different moisture regimes.

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3.7 Supplementary material

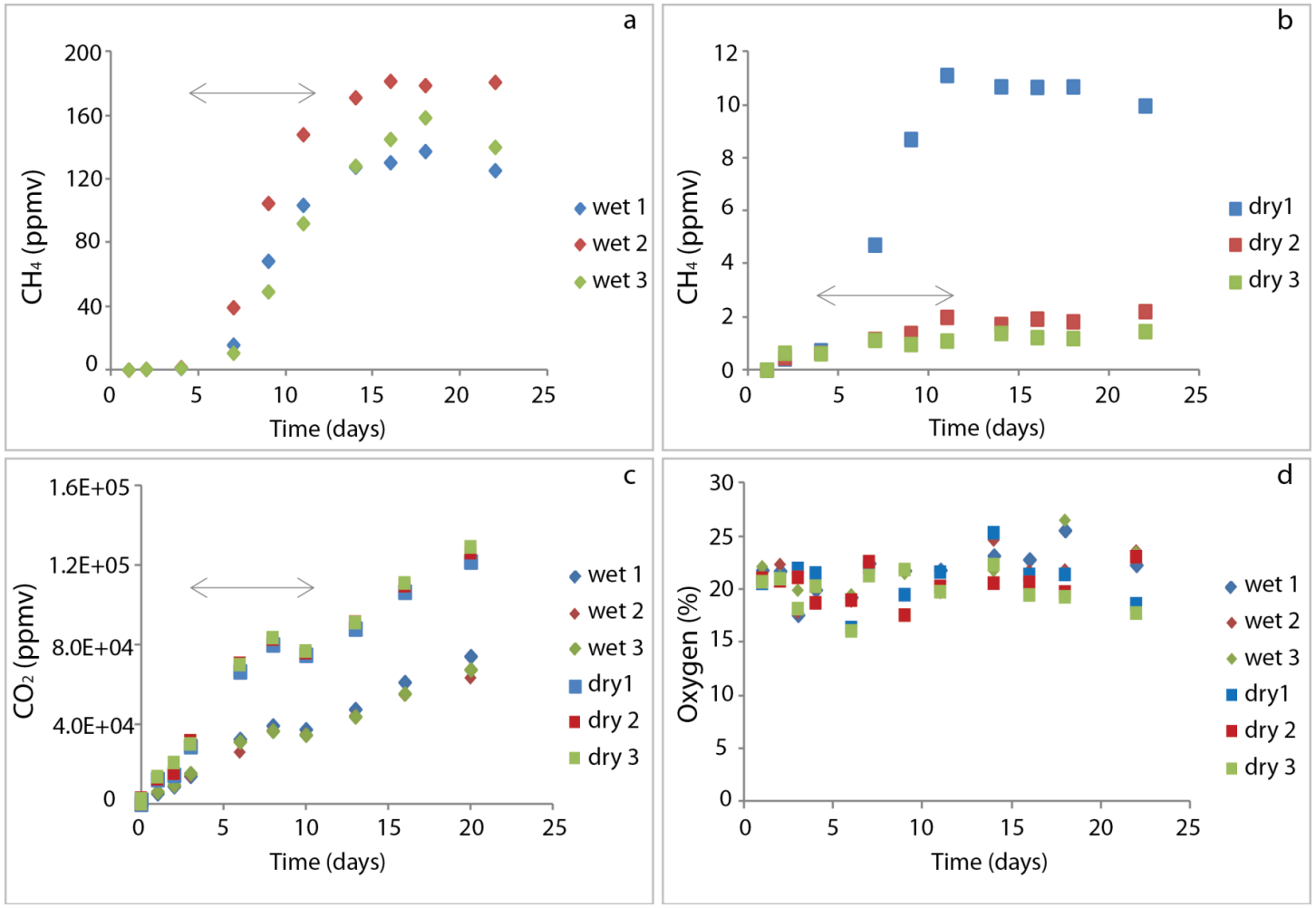

Figure S3.1 CH_4 (a,b), CO_2 (c) and oxygen (d) concentration in the headspace of flasks with tank slurry sampled from *G. squarrosa* incubated at two different moisture levels (wet, dry) for 21 days. Arrows indicate the period used for determination of gas production rates. Treatments are given in triplicates (wet 1-3, dry 1-3).

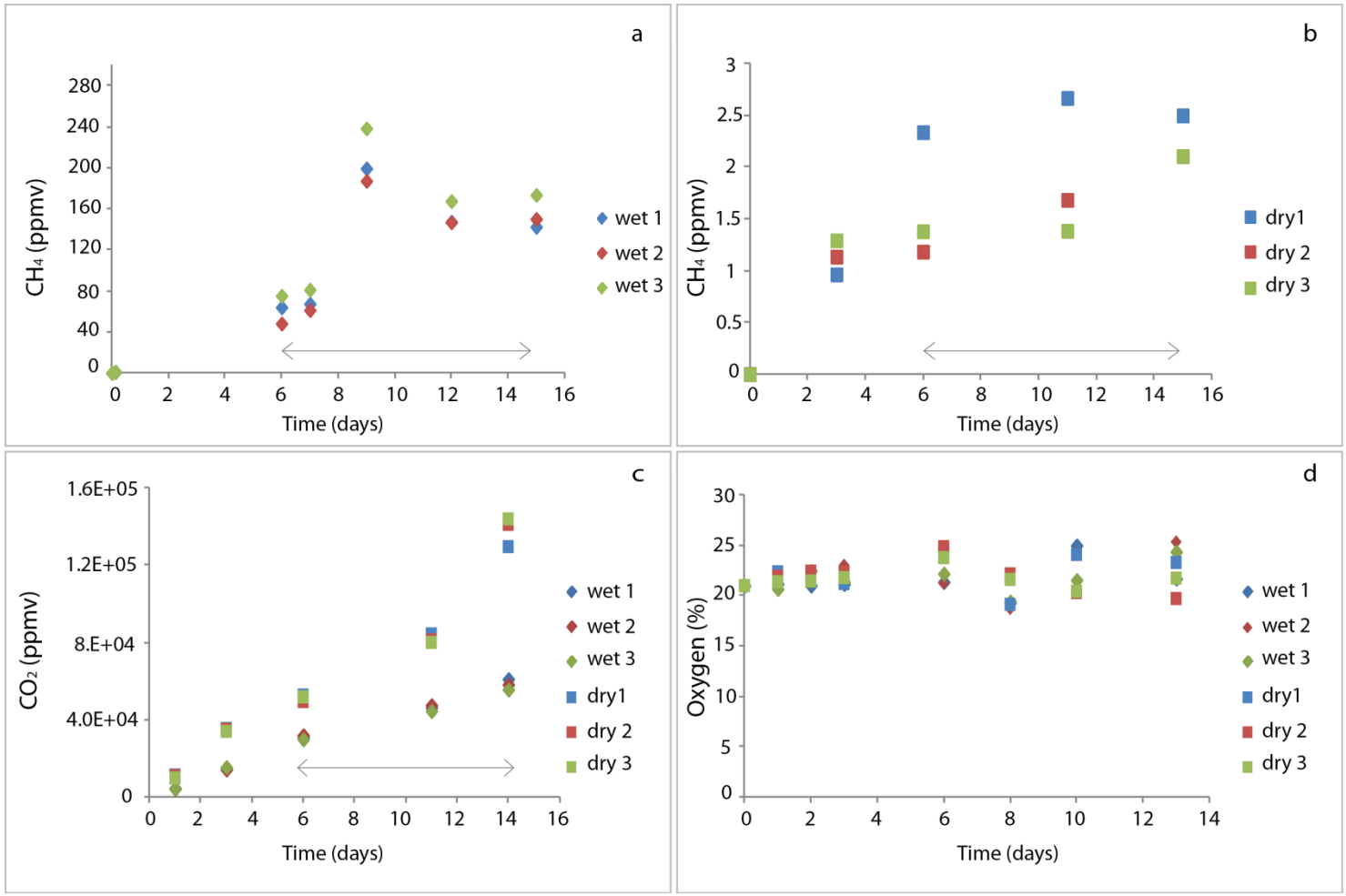

Figure S3.2 CH_4 (a,b), CO_2 (c) and oxygen (d) concentration in the headspace of incubation flasks with tank slurry from *G. gloriosa* incubated at two different moisture levels (wet, dry) for 15 days. Arrows indicate the period used for the determination of gas production rates. Treatments are given in triplicates (wet 1-3, dry 1-3).

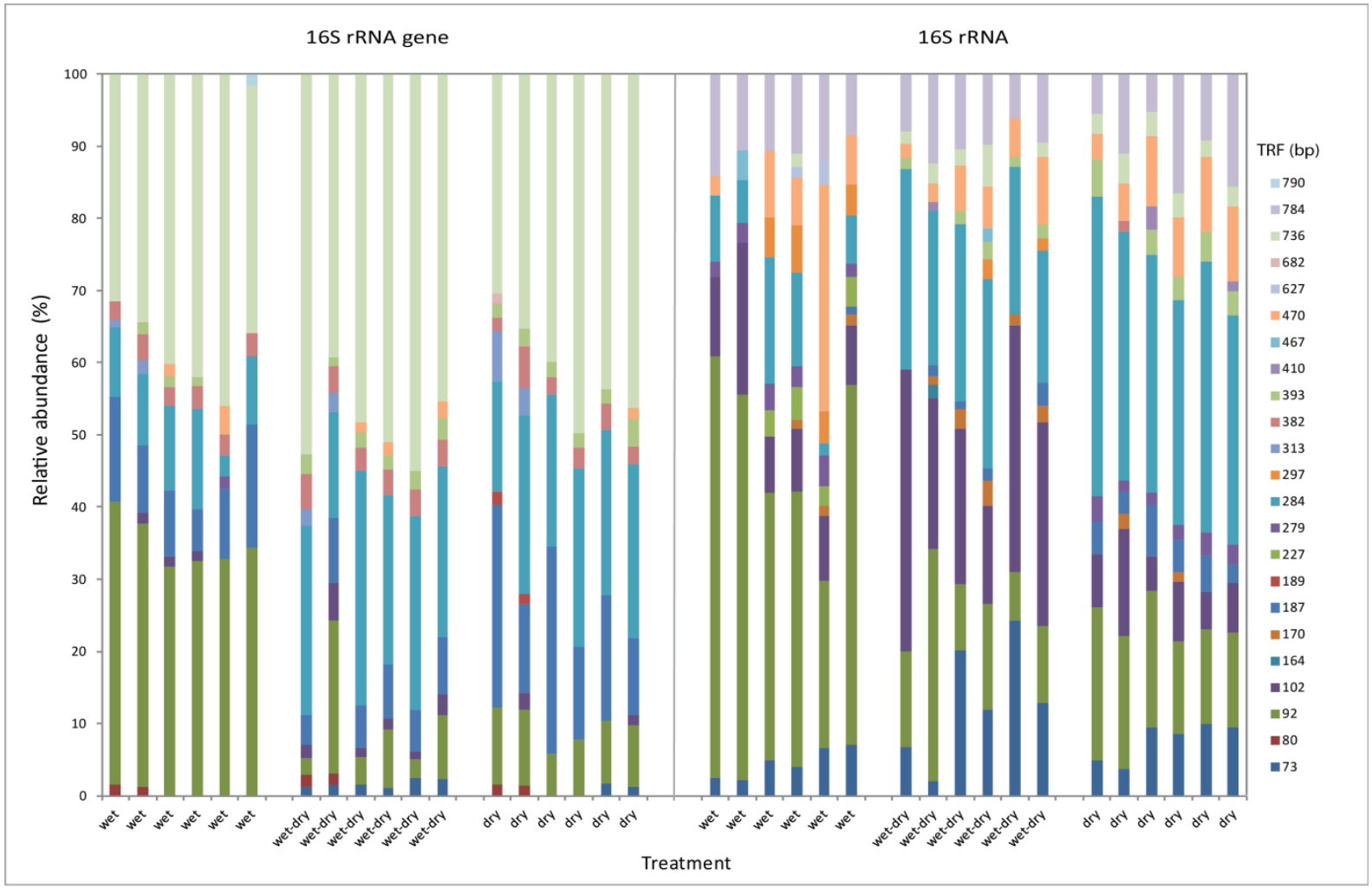

Figure S3.3 TRFLP profile of the archaeal community in tank bromeliad slurry of *G. squarrosa* under three different water amendments (wet, wet-dry, dry). The graph shows the relative abundances of TRFs as a measure of the community composition by targeting the archaeal 16S rDNA (left) and the 16S rRNA (right).

4. Drying effects on the bacterial community in tank bromeliad slurry incubated inside and outside of bromeliad tanks

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Contributions
FBB designed the study, cultivated bromeliads in the greenhouse, performed greenhouse-experiment, performed lab work (incubation outside of bromeliads: nucleic acid extraction, 454 pyrosequencing PCR; incubation inside of bromeliads: *nifH* qPCR, 454 pyrosequencing PCR), evaluated all data, performed statistical analysis and wrote the manuscript
BP performed lab work (incubation inside of bromeliads: nucleic acid extraction, 16S qPCR, T-RFLP)
GOM provided tank slurry, designed the study, cultivated bromeliads in the greenhouse, performed greenhouse experiment and wrote the manuscript
RC wrote the manuscript

4.1 Abstract

Epiphytic bromeliads increase the volume of arboreal carbon storage in neotropical forests by creating catchments between their leaves in which water and litter accumulate (=tank slurry). The organic matter in this slurry can be anaerobically decomposed by microorganism inhabiting the tank and result in the release of nutrients to the plant and of CH_4 into the atmosphere. In a previous study we showed that the availability of water controlled the archaeal community composition and the methanogenic pathway in the tank slurry. In the present study we investigated the effect of water availability on the resident and active bacterial community by targeting 16S rDNA and 16S rRNA, respectively. The bacterial community was analyzed with respect to abundance, using quantitative PCR, and composition, using T-RFLP (terminal restriction fragment length polymorphism) and 454 pyrosequencing. For comparison, the bacterial community composition was also analyzed in the soil from the site where the bromeliad tank slurry was sampled and after incubation in glass vials outside of bromeliads.

Inside the bromeliad, the bacterial community size in the tank slurry stayed constant but the relative abundance of *Burkholderiales*, mainly represented by the genus *Burkholderia*, more than tripled and the bacterial diversity decreased when water availability decreased. Copy numbers of *nifH*, a marker gene for nitrogen fixation, increased in parallel with the relative abundance of *Burkholderia*. The relative abundance of *Acidobacteria*, *Actinomycetales* and *Sphingobacteriales* was significantly higher after incubation of the tank slurry inside than outside of the bromeliad. However, the *Burkholderia* was the most dominant group in tank bromeliad slurry irrespectively of incubation conditions.

4.2 Introduction

Tropical forests are known as hot spots of world's biodiversity (Myers *et al*., 2000). Their vegetation can be divided into different strata, from the soil up to the canopy and each layer provides specific habitat conditions. For instance, 'arboreal tropical soils', including the debris within bromeliad tanks revealed to have a higher total organic carbon and nitrogen content than the soil on the ground (Pittl *et al*., 2010; **Chapter 2**). The debris within the bromeliad tanks provide a unique habitat for many different aquatic and terrestrial (micro)organisms (Kitching, 2001; Goffredi *et al*., 2011b). These microorganisms aerobically and anaerobically decompose the accumulated organic material and release nutrients for the plant. They also produce methane (CH_4) and thus contribute to the neotropical CH_4 cycle (Martinson *et al*. 2010). Various taxa of hydrogenotrophic and aceticlastic methanogenic archaea were detected in the slurries of Ecuadorian and Costa Rican bromeliad tanks (Martinson *et al*., 2010; Goffredi *et al*., 2011a; **Chapter 3**, Brandt *et al*., 2014).

Nevertheless, studies of microbial communities and their contribution to carbon (e.g. methanogenesis) and nitrogen (e.g. N_2 fixation) cycling in tank bromeliads are still rare. Nitrogen-fixing microorganisms may contribute to the nutrition of the nitrogen-limited tank bromeliads (Brighigna *et al*., 1992; Leroy *et al*., 2009).

Factors that influence the communities in tank bromeliads are assumed to be substrate quality and quantity, tank morphology, degree of anoxia, pH and desiccation (Goffredi *et al*. 2011a). Although the humid tropics are characterized by high annual rainfall, tank bromeliads can face periods of restricted water availability. Rainless periods of some hours can result in water stress for bromeliads, and small plants can dry out completely (Zotz and Thomas, 1999). Drought periods are anticipated to increase in tropical forests based on climate change scenarios (Cox *et al*., 2008; Malhi *et al*., 2008), so that water availability may indeed be an important controlling factor in-situ.

In a previous experiment we have shown that water is a major determinant for the composition of the methanogenic archaeal community and the methanogenic pathway in tank bromeliad slurry (**Chapter 3**, Brandt *et al*., 2014). However, methanogenesis is the last step in the anaerobic degradation of organic matter, which consists of a cascade of anaerobic processes involving hydrolysis of organic polymers (e.g chitin, polysaccharides) and fermentation of monomers (e.g. sugars) to carbon dioxide, hydrogen and acetate,

which are then used by methanogenic archaea to produce CH_4. The hydrolysis of polymers and the fermentation processes can be provided by several microbial guilds that are common in anoxic soils and sediments (Le Mer and Roger, 2001; Liu and Whitman, 2008). In fact, common soil bacterial phyla (*Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Firmicutes*) have been detected in tank bromeliads, and it has been shown that the bacterial abundance abundance is higher in the tank slurries than in the adjacent soils on the ground (Pittl *et al*., 2010). However, the effect of drought conditions on the bacterial community in tank slurries has not yet been studied.

Therefore, we investigated the effect of different water amendments on the abundance and community composition of bacteria in tank bromeliad slurry using the same experimental set-up as in Brandt et al. (2014). We also investigated the bacterial community in tank slurry incubated in glass vials outside of bromeliads. This experiment was established to check a possible effect of the plant on the bacterial community. Since plants are known to influence the microorganisms in the rhizosphere by root exudation (Rovira, 1969), an analogous effect may occur in epiphytic tank bromeliads. Bromeliads absorb water and nutrients almost exclusively via specialized trichomes from the tank (Benzing and Renfrow, 1974). Therefore it is assumed that they also secrete compounds into their tanks as shown for *Vriesea gigantea* (Inselsbacher *et al*., 2007) affecting the inhabiting organisms.

4.3 Material and methods

Experimental setup

Incubation inside of bromeliad tanks

The same experimental set-up was used as in Chapter 3. In brief, the organic tank slurry for this experiment originated from a single plant of the species *Guzmania squarrosa*, sampled in Ecuador in the south of Loja (adjacent to Podocarpus National Park, detailed site description see Martinson *et al*., 2013) at 2000 m height in May 2011. The tank slurry is defined here as organic matter and water located in the various leaf axils. The slurry was completely sampled and pooled (348 g). Further, three samples were taken from nearby soil and all samples were immediately transported in a cooled state (4°C) to the Max-Planck-Institute for Terrestrial Microbiology in Marburg, Germany and stored at 4°C. Tank slurry was homogenized and 9 g were filled into each central tank of 9 bromeliads of the species *Aechamaea fasciata*. Tank bromeliads of *Aechamaea fasciata* "Primera" were of 10 cm size and cultivated with empty tanks by Corn.Bak® B.V. (Netherlands) in the greenhouse. The plants belonged to the same growth stage of 1 year age. Plants of *A. fasciata* have the advantage to posess one stable central tank even at an early growth stage. This central tank allows precise water amendment and sampling.

The experiment was performed in the greenhouse at a temperature of 25 ± 3°C for 28 days. Different amounts of water were spread onto the slurry located in the central tank. Three plants were watered daily by spreading 2 mL deionized water in the central tank (treatment P1), three plants were watered once per week with 2 mL of deionized water (treatment P2) and three plants were not watered and slowly dried over the period of 28 days (treatment P3).

Subsequently, the tank slurry was sampled for molecular analysis. The leaves were carefully detached and the tank slurry was homogenized. For each plant, 2 samples of 0.3 g (fresh weight) tank slurry were taken with a sterile spatula and stored at -80°C till nucleic acid extraction. In total, 6 samples were analyzed per treatment. Total carbon and nitrogen concentration of tank slurries were analyzed on a CHNS-elemental analyzer (Analytical Chemical Laboratory, University of Marburg).

Molecular analysis

Total nucleic acids were extracted from 0.3 g tank slurry and soil samples using a phenol-chloroform extraction described in Chapter 3. If not otherwise described the following molecular analyses were performed to investigate the resident and active bacterial community in the tank slurry targeting the 16S rDNA as well as the 16S rRNA, respectively.

Terminal restriction fragment length polymorphism (TRFLP) was performed targeting bacterial ribosomal 16S rDNA and reversely transcribed 16S rRNA using the primer combination 27f/907r (Osborne *et al*., 2005; Muyzer *et al*., 1995) with a 6-carboxyfluorescein (6-FAM) labeled forward primer. The purified amplicons were digested for 15 min at 37°C using *MSPI Fast Digest* enzyme (Fermentas). The fragmented DNA was purified using the SigmaSpin™ post-reaction clean-up columns (Sigma-Aldrich) following manufactures' manual. The size separation was conducted on an ABI PRISM 3130 capillary Genetic Analyzer (Applera Deutschland GmbH) using the software Genescan 4.0 (Applied Biosystems). The TRFLP data were obtained in comparison with an internal DNA standard and the resulting TRFLP profiles were standardized as described in Dunbar et al. (2001) using the peak area.

Absolute numbers of bacterial 16S rDNA and 16S rRNA copies were determined by quantitative PCR (qPCR) using the primer combination Ba519f/Ba907r (Stubner, 2002). The qPCR was set up in 96-well micro titer plates (BioRad). Each qPCR reaction was set up in a total volume of 25 µl 1x SYBR® Green Ready Mix™ (Sigma), 3 mM $MgCl_2$ (Sigma), 0.66 µM of each primer and 1 µM FITC (fluorescein isothiocyanat, BioRad) as well as 2 µl of template. Purity of reagents was checked by negative controls which were run in parallel without matrix DNA. For absolute quantification a dilution series was prepared with 10^1 – 10^7 gene copies using a standard that originated from a clone containing a bacterial 16S rRNA gene as a plasmid insert. The following PCR program was used: 94°C for 8 min followed by 50 cycles of 94 °C for 20 s, 50 °C for 20 s, 72°C for 50 s. The quantification of *nifH*, a marker gene for nitrogen-fixing bacteria, was done using the primer combination PoIF/PoIR (Poly *et al*., 2001). Quantitative PCR was performed after Mao et al. (2011). Afterwards melting curves were performed to ensure purity of PCR products.

PCR-based 454 pyrosequencing was applied to investigate the bacterial community in the tank slurries and soil samples. A subunit of the bacterial 16S rDNA was amplified using the primer F515 and R806, described by Bates et al. (2011). The F515 primer contained a Roche 454-A pyrosequencing adapter (Roche Applied Science, Branford, CT, USA), a 'GTG' linker sequence and an incorporated 6-bp barcode sequence which is unique for each sample. The amplifications were conducted in a total volume of 50 µl each containing 2 µl (15 µM concentration) of forward and reverse primers, 5 µl of 10x AccuPrime PCR Puffer (Invitrogen), 1 µl AccuPrime Taq DNA Polymerase (Sigma-Aldrich) and 1 µl of template DNA. Amplification was performed using the following thermoprofile: 5 min at 94 °C followed by 35 cycles of 94 °C, 30 s; 50 °C, 30 s; 68°C, 30 s; 94°C 30 s; 68 °C, 10 min. Pooled duplicate reactions were purified using the GenElute™ PCR Clean-Up Kit (Qiagen) and quantity was checked via DNA Qubit (Life technologies). One single sample was prepared that contained all barcoded PCR products in equal amounts to ensure equivalent sequencing from all samples. The sample was sent for sequencing at Max Planck Genome Centre in Cologne. Data analysis was performed using the mothur software package version 1.31.2 (http://www.mothur.org/) following the standard operational procedure (SOP, Schloss *et al*., 2011). The two replicate samples of each plant and the three replicates of the soil samples were grouped and analyzed as one sample. In total, three replicates per treatment (P1, P2, and P3) were processed as follows. In brief, all reads with an average quality score above 25 and minimum read lengths of 200 bp were accepted. Trimmed sequences were aligned against the Silva bacterial 16S rRNA database. Reads classified as Chloroplast, Mitochondria or 'unknown' were removed. Chimeric sequences were removed using the implementation of Chimera-uchime. Sequences were clustered into operational taxonomic units (OTU) by a 0.03 distance level. The alpha diversity of the samples was calculated by the collect.single command which generated the Chao1 richness estimators and the inverse Simpson diversity index. Finally, the quality-filtered dataset contained in total 87,782 sequences.

Incubation outside of bromeliad tanks

An incubation of tank slurry was conducted without plants. Therefore, 9 g of tank slurry was filled into 27-mL darkened glass vials to artificially simulate bromeliad tanks. Samples were treated in the same way as described above. In brief, in three vials 2 mL water was spread every day (treatment C1), in three vials 2 mL water was spread once per week (treatment C2) and three samples of slurry were air dried in the vials (treatment C3). After the incubation of 28 days the slurry was sampled for nucleic acid extraction. For each vial, two samples of 0.3 g (fresh weight) slurry were taken with a sterile spatula and stored at -80°C till nucleic acid extraction as described before. Water contents were determined gravimetrically by drying 1 g aliquot of each tank slurry at 65°C for 72 h. PCR-based 454 pyrosequencing was performed as described above. Finally, the quality-filtered dataset contained in total 75,870 sequences.

Comparison of incubation treatments

Bacterial community compositions were compared between treatment P1 and treatment C3 since these incubations had similar gravimetric water contents (26% - 29%). The 40 most abundant OTUs (in total sequences assigned to the OTU) of both treatments were grouped according to their phylogenetic assignment at order level. These 40 OTUs covered 50% and 42% of the total sequences of treatment C3, respectively P1. The relative abundance of the phylogenetic groups between the treatments was directly compared using ANOVA (see Statistical analysis).

In all treatments the order of *Burkholderiales* represented the dominant bacterial group. Therefore, the 20 most abundant OTUs assigned as *Burkholderiales* were chosen from treatment P3 as well treatment C1 showing the highest relative abundance of *Burkholderiales*. The get.oturep command in mothur was used to generate a fasta-formatted sequence file containing one representative sequence for each of the 20 most abundant OTUs, in total 40. The representative 40 16S rDNA sequences were aligned and added to the Silva 115 reference tree (Quast *et al*., 2012). A neighbour-joining tree was constructed by bootstrap analysis of 1000 replicates.

Statistical analysis

All statistical analyses were conducted in R version 2.10.1. The effect of different water amendments on the bacterial abundance (16S rDNA and 16S rRNA copies), on the abundance of *nifH* gene copies as well as on the bacterial diversity, using Chao1 and InvSimpson indices, was assessed using the one-way analysis of variance (ANOVA) and the *post hoc* Tukey (HSD) test. Equally, differences in the relative abundances of OTUs in selected from treatment P3 and C1were determined using ANOVA. All data were checked for normal distribution and homoscedasticity using Kolmogorov–Smirnov test and Levene's test, respectively. ANOVA was done using the stats package. The impact of moisture on the bacterial community based on TRFLP results and 454 pyrosequencing data (OTU based) was analyzed using non-metric multidimensional scaling (NMDS) and *permutational* multivariate analysis of variance (Permanova). Permanova and NMDS analyses were done using package 'vegan'. Species indicator analysis reveal species, here OTUs, that are specific for a habitat type, for environmental conditions, or environmental changes. The 200 most abundant OTUs were selected for the species indicator analysis which was performed using the 'multipatt' function of the R package 'indicspecies'. All levels of significance were defined at $P \leq 0.05$.

4.4 Results

Incubation inside of bromeliad tanks

Gravimetric water content and oxygen (O_2) concentration were already determined in **Chapter 3**. In brief, gravimetric water content was 29 ± 1% in treatment P1, 6 ± 3% in treatment P2 and 1 ± 0.5% in treatment P3. Microsensor measurements revealed that O_2 concentrations decreased from atmospheric concentration at the surface of the tank slurry to undetectable concentrations at 2-3 mm depth and below in the treatments P1. However, tank slurry of treatments P2 still showed an atmospheric O_2 concentration at 3.5 mm depth. Carbon and nitrogen concentration were 46.5 ± 0.4% and 2.06 ± 0.04% in treatment P1; 46.6 ± 0.3% and 2.1 ± 0.06% in treatment P2 and 46.6 ± 0.2% and 2.06 ± 0.04% in treatment P3, respectively.

Bacterial 16S rDNA copy numbers remained constant under the tested water amendments. Albeit not significant, a slight increase of 16S rRNA, targeting the more active members of the bacterial community, was observed in tank bromeliad slurry upon drought (Figure 4.1a). The abundance of the *nifH* gene significantly increased with increasing drought in the tank slurry from 6×10^8 gene copy numbers gdw^{-1} in treatment P1 to 2×10^9 gene copy numbers gdw^{-1} in treatment P2. Treatment P2 and P3 did not differ in their *nifH* gene copy numbers (Figure 4.1b).

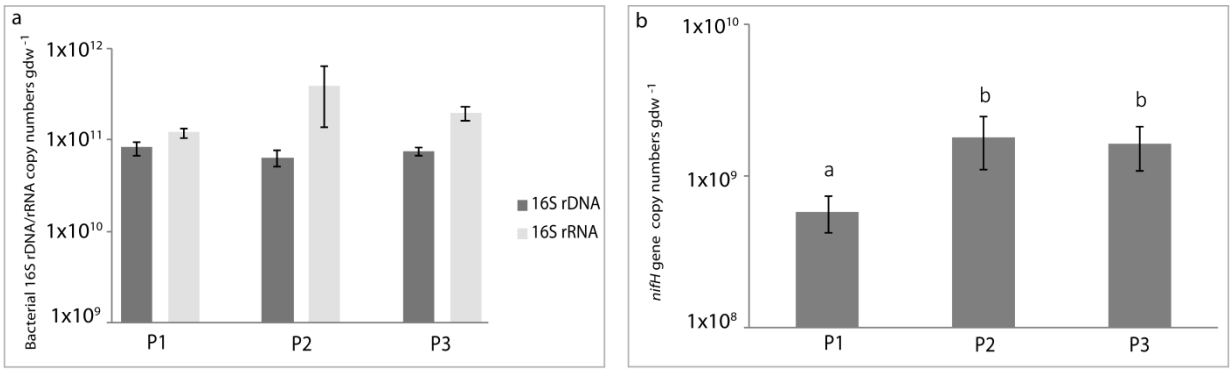

Figure 4.1 Copy numbers of bacterial 16S rDNA and 16S rRNA (a) and *nifH* gene (b) in tank bromeliad slurry incubated in plants under different moisture contents (P1, P2, P3) after 28 days of incubation. Error bars represent standard deviation (n=3). Different letters indicate significant differences.

The effect of moisture on the bacterial community incubated in bromeliad tanks is displayed in NMDS plots (Figure 4.2a-c). Community profiles based on T-RFLP data,

targeting the resident bacterial community, significantly differed between all treatments (Figure 4.2a). Based on 16S rRNA profiles community pattern of treatment P1 were different to treatment P2 and P3 but did statistically not differ between P2 and P3. The community profile based on 454 pyrosequencing data was significantly affected by the different water amendments. NMDS analysis showed a distinct clustering of bacterial communities according to their treatment (OTU based; Figure 4.2c).

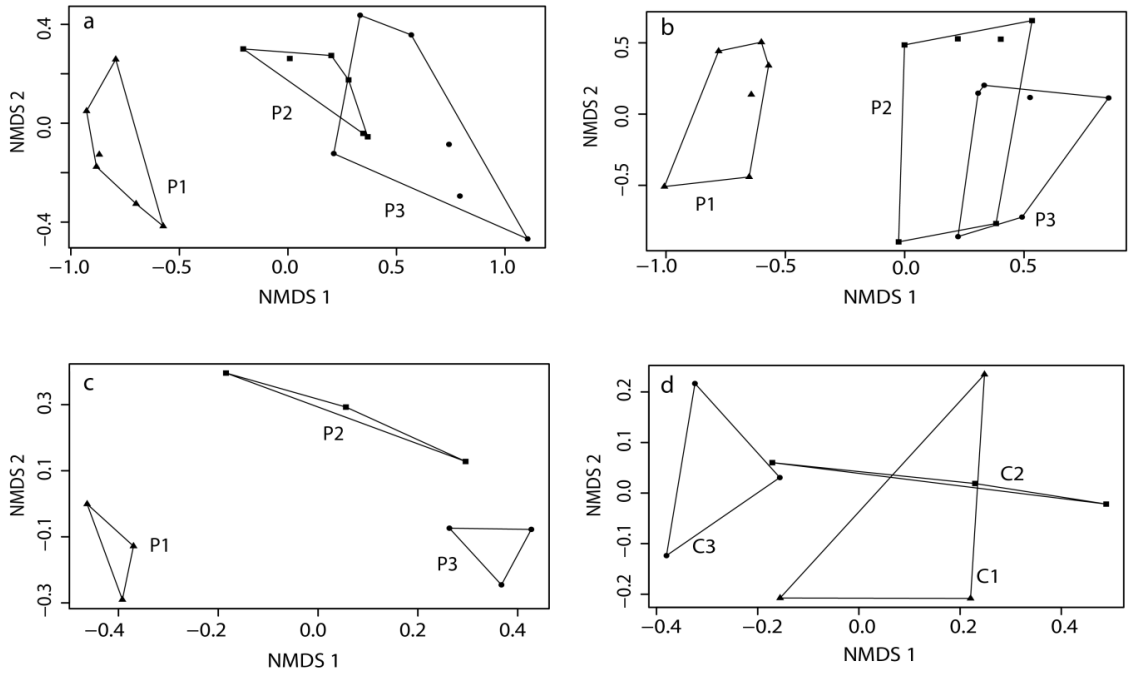

Figure 4.2 Non-metric multidimensional scaling (NMDS) plots of bacterial communities present in tank bromeliad slurry incubated in plants (a, b, c) and glass vials (d) under three different water amendments (P1/C1 daily watered, P2/C2 watered once per week, P3/C3 without irrigation). Community patterns are based on TRFLP data of 16S rDNA (a), 16S rRNA (b) and 454 pyrosequencing data (16S rDNA, OTU based, c, d). NMDS plots based on Bray–Curtis distances with stress of 5.5% for a, 10.4% for b, 5.8% for c and 6.8% for d. Different symbols denote the different treatments, while samples of the same treatment were graphically grouped.

In order to identify bacterial lineages which were affected by drought we used 454 pyrosequencing targeting the bacterial 16S rDNA (Figure 4.3A). In general, bacterial communities in tank slurry were dominated by *Actinobacteria, Acidobacteria*, *Bacteroidetes* and *Proteobacteria*. Several trends were observed: The *Acidobacteria* and the *Bacteroidetes* significantly decreased upon drought. The *Acidobacteria* were dominated by *Acidobacteria* GP1 and the *Bacteroidetes* mainly by *Sphingobacteriales.* They both decreased by half from treatment P1 to P2 and again to treatment P3 (Figure

4.3Ac). Within the *Sphingobacteriales* most sequences (>85%) were assigned to the genus of *Mucilaginibacter* in all treatments (data not shown). In contrast, the *Proteobacteria* significantly increased upon drought, i.e., from 53 ± 2% in treatment P1 to 71 ± 7% in treatment P2 to 82 ± 2% in treatment P3 (Figure 3Aa). At the class level *Betaprotobacteria* increased and represented the most prominent group under low moisture (Figure 3Ab). Deeper phylogenetic analysis revealed *Burkholderiales* as the dominant taxon within the order of the *Betaproteobacteria* in desiccated tank slurry (Figure 3Ac). More than 97% of the *Burkholderiales* sequences were assigned as genus *Burkholderia* in all treatments (data not shown). The same results were obtained using an OTU-based analysis (Figure 3.4). OTU 1, assigned to the genus *Burkholderia*, showed the highest abundance in tank slurry of low water content. Species indicator analysis further revealed that OTU 6, assigned as *Burkholderia* sp., was specific for treatment P2 and P3 (P= 0.005).

The high coverage of 454 data indicated that a large part of the diversity in all samples had been captured. The average coverage was 91 ± 2 % (s.d., n=3). Different values of estimator OTU abundance, Chao1 and InvSimpson index were obtained for tank bromeliad substrate samples under different moisture levels revealing differences in bacterial diversity (Table 4.1). In the tank substrate with the highest moisture the bacterial community showed the highest values of InvSimpson and Chao1 indices indicating highest diversity. With increasing drought the bacterial diversity decreased.

Table 4.1 Alpha-diversity indices based on 454-pyrosequencing data of 16S rDNA fragment sequences in tank bromeliad slurry incubated in plants under different water amendments. Values are means ± standard deviation (n=3). Different letters indicate significant differences.

Treatment	Coverage	No. Seqs	No. OTUs	InvSimpson	Chao1
P1	89 ± 3%	11244 ± 2661	1964 ± 769	44 ± 15[a]	3659 ± 847[a]
P2	91 ± 2%	11480 ± 3210	1505 ± 596	4 ± 2[b]	2651 ± 362[a]
P3	94 ± 0.4%	15074 ± 2140	1391 ± 264	2 ± 0.2[b]	2451 ± 238[a]

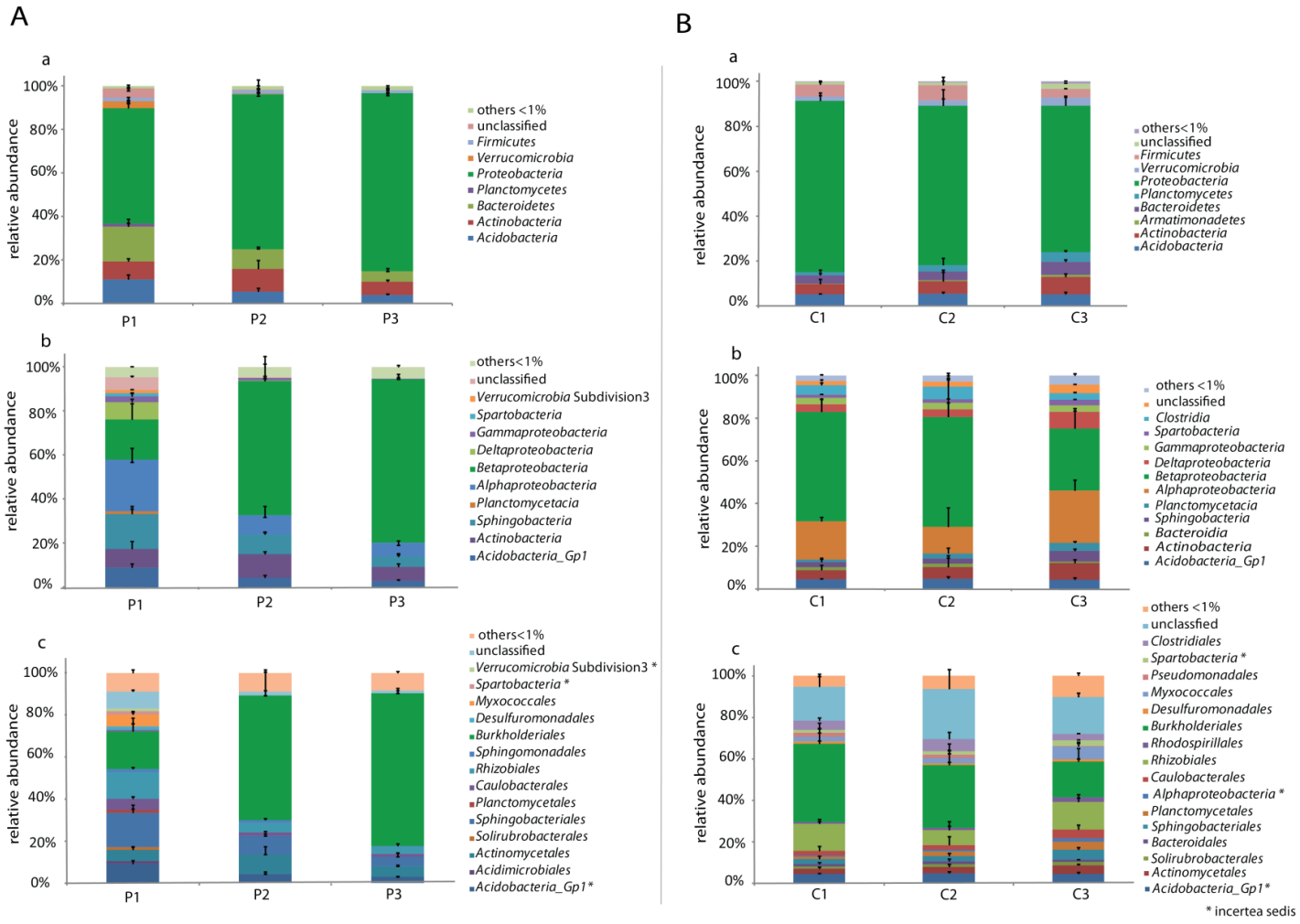

Figure 4.3 Relative sequence abundance (%) of bacterial groups (>1 %) at phylum (a), class (b) and order (c) level in tank bromeliad slurry incubated in plants (A) or in glass vials (B) under three different water amendments (P1/C1 watered daily, P2/C2 watered once per week, P3/C3 without irrigation) determined by 454 pyrosequencing targeting the 16S rDNA (n=3).

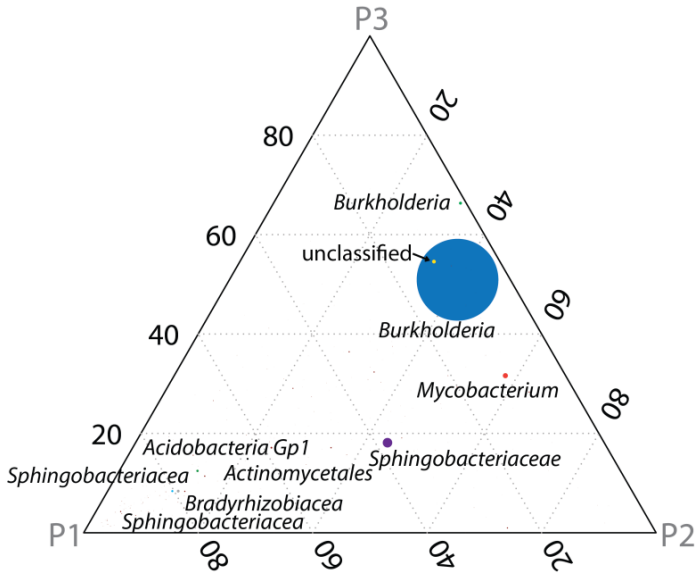

Figure 4.4 Ternary plot of OTU based 454 pyrosequencing data targeting the bacterial 16S rDNA in the tank slurry incubated in plants under different water amendments (P1, P2 and P3). Each circle represents one OTU. The size of each circle represents its relative abundance. Each corner of the triangle represents a proportion of 100% for the respective treatment with the other corners representing 0% of that treatment. As the relative abundance of an OTU in a treatment increases then it moves towards the corner representing that treatment. The 10 most abundant OTUs are named.

Soil sample

The water content of the soil samples ranged between 1% to 2%. During sequence processing the sequences of the three soil samples were combined and therefore, a statistically valid comparison with the tank communities is not possible. However, the objective was to obtain information of the main bacterial phylotypes in the soils. The bacterial phyla found in the soil were the same as those found in the tank slurry, but the relative abundance of the phyla was different. The *Acidobacteria* and *Proeteobacteria* were the most dominant groups in the soil samples (Figure 4.5). In the soil, the *Acidobacteria* were with ~32% relative sequence abundance ten times more abundant than in the tank slurry under dry conditions (Figure 4.3Aa, P3). Contrary, the *Proteobacteria* were less abundant in the soil samples (~ 40% relative sequence abundance) than in the desiccated tank slurry (~ 82% relative sequence abundance; Figure 3Aa, P3). The *Actinobacteria* exhibited with ~ 8% relative sequence abundance in the soil samples a similar relative abundance than in the bromeliad tank slurry, whereas the

Bacteroidetes (~ 2% relative sequence abundance) were less and the *Planctomycetes* (~ 6% relative sequence abundance) more abundant in the soil than in the tank slurry samples.

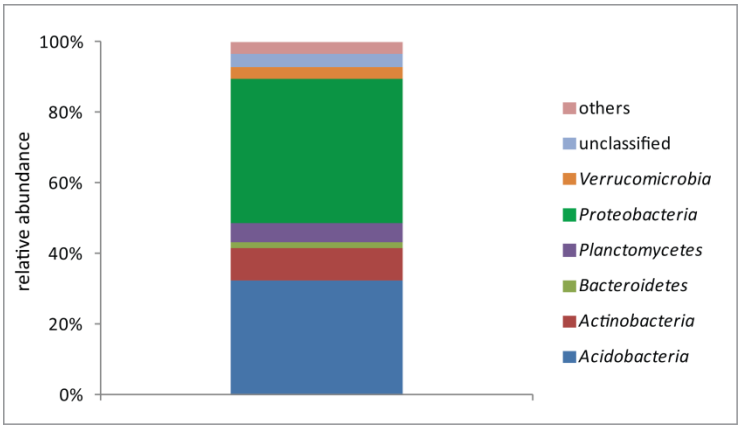

Figure 4.5 Relative sequence abundance (%) of bacterial phyla in soil samples determined by 454 pyrosequencing targeting the bacterial 16S rDNA (n=1).

Incubation outside bromeliad tanks

Tank slurries, which were incubated in vials outside the bromeliads and were watered during the incubation time (treatment C1 and C2), became permanently flooded in the vials and their water content was comparable with 48 ± 2%. The slurry, which was incubated in the glass vials without any irrigation (treatment C3) had a water content of 26 ± 1.8%. Based on the pyrosequencing results a high coverage was observed for all samples, but the bacterial diversity was lower in the treatments C1 and C2 than in C3 (Table 4.2). Differences in the bacterial community composition between the treatments, although statistically not significant, are displayed in a NMDS plot using 454 pyrosequencing data of bacterial 16S rDNA (Figure 4.2d). The *Burkholderiales* were the most dominant group in the slurries that were incubated in glass vials, with highest proportion under flooded (C1, C2) than non-flooded (C3) conditions (Figure 4.3B).

Table 4.2 Alpha-diversity indices based on 454-pyrosequencing data of 16S rDNA fragment sequences in tank bromeliad slurry under different water amendments incubated in glass vials. Values are means ± standard deviation (n=3). Different letters indicate significant differences.

Treatment	Coverage	No. Seqs	No. OTUs	InvSimpson	Chao1
C1	89 ± 2%	11375 ± 3311	1688 ± 107	12 ± 7[a]	4847 ± 121[a]
C2	90 ± 1%	14182 ± 2850	1898 ± 278	16 ± 16[ab]	5798 ±689[a]
C3	89 ± 1%	14066 ± 4277	2215 ± 719	44 ± 14[b]	6769 ± 2496[a]

Comparison of treatment C3 and P1

To check for a possible effect of the plant on the bacterial community composition, samples of treatment C3 (incubated in glass vials) and samples of treatment P1 (incubated in plants) were compared (Figure 4.6), since these samples exhibited a similar water content and a similar bacterial diversity (Table 4.1, Table 4.2). Analysis of the 40 most abundant OTUs revealed significant differences in the relative abundances of single bacterial orders. *Acidobacteria*, *Actinomycetales* and the *Sphingobacteriales* were significantly higher in the slurries that were incubated inside the bromeliad tanks, while several other bacterial orders, with minor relative sequence abundance, were significantly higher in the slurries incubated outside of the bromeliad tanks (e.g. *Armatimonadales*, *Bacteroidales*, *Clostridiales*, *Pseudomonadales*, *Rhodospirales*, *Sphingomonadales*). The order *Burkholderiales* was in treatment C3 and P1 the most dominant bacterial group with a relative abundance of more than 12%. A further prominent group was represented by the *Rhizobiales,* of which the abundance did not differ between the tank slurries incubated inside or outside of the bromeliad tanks.

Regardless of any treatment the order *Burkholderiales* was the most represented group in tank bromeliad slurry. No clustering according to their treatment was observed in a phylogenetic tree using the representative sequences of the 20 most abundant OTUs assigned as *Burkholderiales* from treatment P3 and C1 (Figure 4.7). The most abundant *Burkholderiales*-OTUs each of treatment P3 and C1, covering 80-90% of all the *Burkholderiales* sequences, clustered remarkable close together (Figure 4.7).

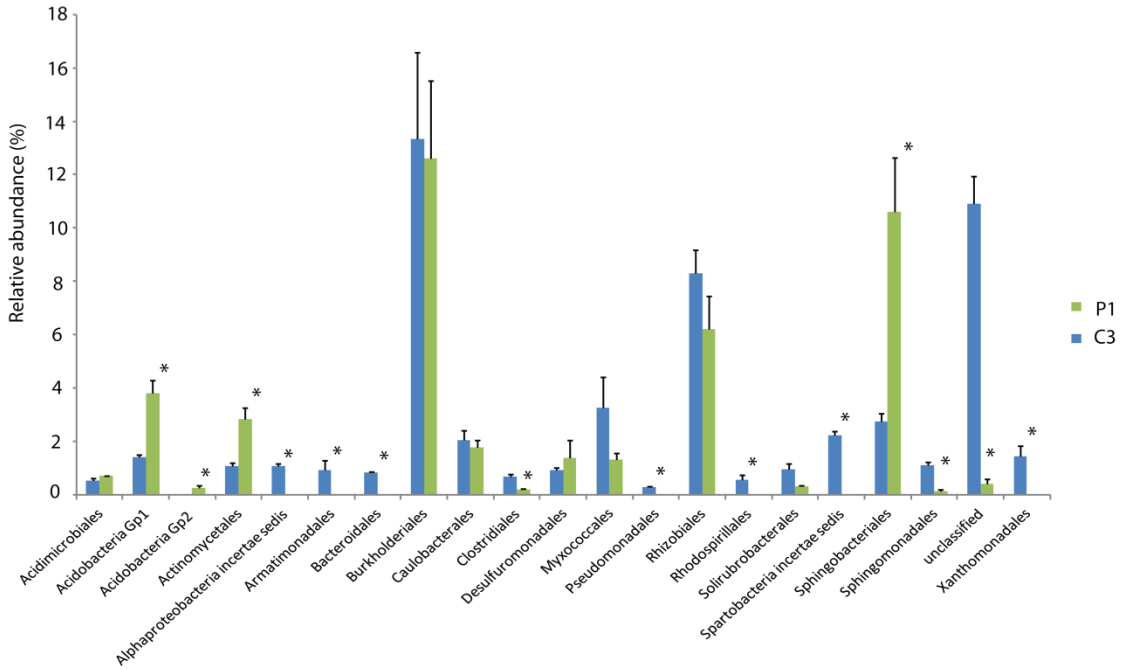

Figure 4.6 Relative sequence abundance of bacterial orders using the 40 most abundant OTUs in tank slurry incubated in a glass vials (C3) or in plant environement (P1) over 28 days. Asterisks indicate significant differences between samples of the two treatments. Error bars represent standard deviations (n=3). OTUs were merged according to their assignment on order level.

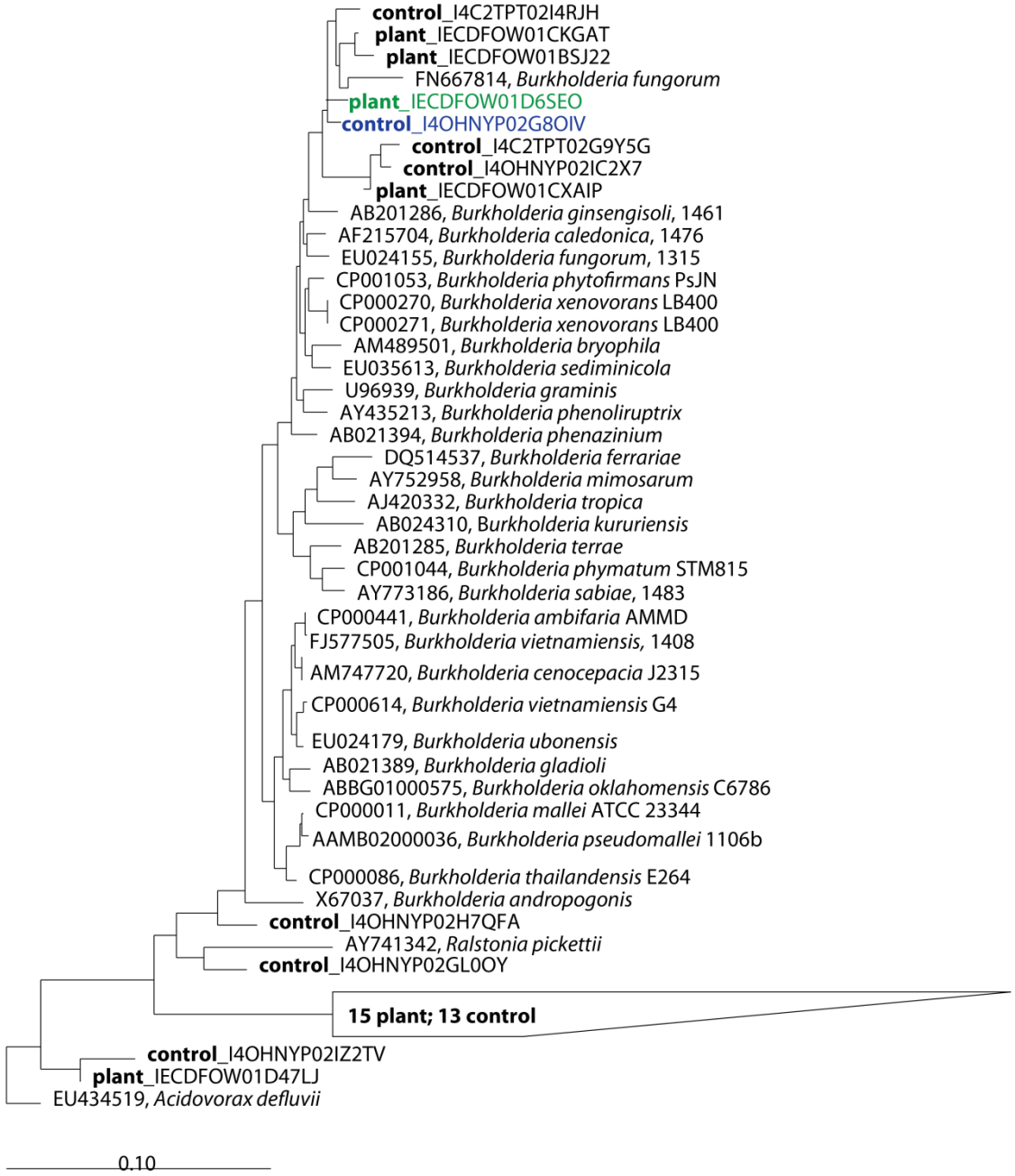

Figure 4.7 Neighbour-joining tree showing the relationships among the obtained 16S rDNA sequences of the 20 most abundant OTUs (bold) from treatment C1 (incubated in glass vials) and P3 (incubated in bromeliad tanks) to sequences of the ARB-Silva reference database. The most abundant OTU in treatment P3 (green) and the most abundant OTU in C1 (blue) covered 80% and 90% of the 20 most abundant OTUs assigned as *Burkholderiales* sequences, respectively. 15 sequences of treatment P3 and 13 sequences of C1 clustered together and were therefore grouped. The scale bar represents 0.1 changes per position.

4.5 Discussion

Incubation inside of bromeliad tanks

Our results showed that water availability played a major role for the composition of the archaeal (**Chapter 3**, Brandt *et al*., 2014) and bacterial communities inhabiting tank bromeliad slurry, as indicated by significant shifts in the community compositions upon drought. By contrast, water availability showed no influence on the size of the bacterial community. However, the 16S rRNA content slightly increased in tank slurries with low moisture. The 16S rRNA likely represents the active members of a microbial community (e.g Egert *et al*., 2011). The increased activity of the bacterial community upon drying can be linked to an increased respiration rate in the same slurry that was higher under an intermediate than under a high moisture level (**Chapter 3**, Brandt *et al*., 2014). A similar response is known for soil ecosystems, where moisture is an important controller of soil respiration (Orchard and Cook, 1983; Curiel Yuste *et al*., 2007; Lu *et al*., 2013). Empirical models support the assumption that under dry conditions soil respiration is low, increases to a maximum at intermediate moisture level and decreases again when moisture content excludes oxygen (Moyano *et al*., 2012). So far, our results indicate that moisture is a determinant for the microbial activity in tank bromeliad slurry.

With increasing drought the relative abundance of *Burkholderia* spp. increased in tank slurry indicating to be more tolerant against drought and oxidative stress than other tank slurry inhabiting bacteria. *Burkholderia* species are described as common soil bacteria but were 100 to 1000 times more numerous on the roots of plants than in the surrounding soil (Coenye and Vandamme, 2007). Lower relative abundance of the *Burkholderia*, in comparison to tank slurry, was also observed in the soil samples (~ 7% relative abundance; data not shown). It is assumed that the *Burkholderia* are well adapted to a plant environment. The plant-microbe-interaction seems to be quite close as some *Burkholderia* spp. even colonize internal tissues (Coenye and Vandamme, 2007). Interestingly, some members of the *Burkholderia* were identified as plant growth promoting bacteria, producing antibiotics to inhibit pathogenic bacterial and fungal growth and fixing nitrogen (El-Banna and Winkelmann, 1998; Pandey *et al*., 2005; Elliot *et al*., 2007; Naveed *et al*., 2014). Especially nitrogen is a limited factor for tank bromeliads. As epiphytic tank bromeliads are entirely dependent on their tank contents for nutrient supply, they receive

nitrogen by mineralization of organic material from the canopy, from atmospheric sources (deposition, Stewart *et al*., 1995), by interactions with animals (Davidson and Epstein, 1989) or probably by microbial N_2 fixation (Brighigna *et al*., 1992).

Gene copies of *nifH*, which is a bacterial marker gene for nitrogen fixation and present in several *Burkholderia* species (Caballero-Mellado *et al*., 2007; Liu *et al*., 2012), increased with increasing drought. However, the microbial nitrogenase enzyme complex is sensitive to O_2. In our study, increasing drought resulted in increased oxygen exposure of the microbial community. Nevertheless, *Burkholderia* species have been shown to fix N_2 even under microaerophilic conditions (Estrada-De Los Santos *et al*., 2001) and to effectively improve the growth of wheat under drought conditions (Naveed *et al*., 2014). Dinitrogen gas-fixing microbes can provide nitrogen in form of ammonium. Interestingly, bromeliads of the species *V. gigantea* take up nitrogen in inorganic and organic forms but showed a considerable nitrogen-uptake preference for ammonium (Inselsbacher *et al*., 2007). Goffredi et al. (2011b) reported the identification of 4 *nifH* sequences from the tank substrates of Costa Rican tank bromeliads (NCBI HQ010120–HQ010123). We determined by BLAST analysis the closest culture relatives to these sequences as *Halorhodospira* sp., *Agrobacter* sp*., Paenibacillus* sp. and the methanogen *Candidatus* Methanoregula boonei. Therefore, we checked the presence of nitrogen fixing genes in methanogens of *Methanosaetaceae* which, equally as the *Burkholderia,* increased in our tank slurry upon drought (**Chapter 3**, Brandt *et al*., 2014). In the genome of *Methanosaetceae concilli* (Barber *et al*., 2011) we found the genes encoding for the nitrogenase subunits (*nifD, nifK, nifH*). Although total nitrogen content of tank slurry in our experiment did not change upon drought, we speculate that N_2-fixing bacteria and/or archaea, presumably members of the *Burkholderia* and/or *Methanosaetaceae* may play a role for the nitrogen supply for tank bromeliads especially under water limited conditions. In general, the detection of *nifH* indicates that tank bromeliads inhabiting microbes are not only involved in carbon cycling (e.g. methanogenesis; Martinson *et al*., 2010; **Chapter 3**, Brandt *et al*., 2014) but also in nitrogen cycling.

In contrast to the *Burkholderia, Acidobacteria* decreased with increasing drought. Twenty six subdivisions are described for *Acidobacteria,* but the knowledge of their metabolic potentials is still quite rudimentarythe because of the difficulty to culture them. *Acidobacteria* are widely distributed in soils and sediments (Barns *et al*., 1999),

wastewater (Crocetti *et al*., 2002), peat bogs (Dedysh *et al*., 2006), acid mine drainage (Kishimoto *et al*., 1991) and hot springs (Hobel *et al*., 2005). The ubiquity of *Acidobacteria* in these habitats supports the assumption that this phylum can use various carbon sources. Data from whole-genome analysis reported that some *Acidobacteria* are versatile heterotrophs capable of using plant polymers and readily oxidizable carbon (Ward *et al*., 2009). Recently, isolation of acidobacterial strains confirmed their ability to degrade plant polymers (Eichorst *et al*., 2011) which may also occur in tank slurry consisting mainly of degraded leaf litter (Stuntz *et al*., 2002). Until today only few anaerobic acidobacterial strains have been isolated (Liesack *et al*., 1994; Coates *et al*., 1999; Pankratov *et al*., 2012). The presence of obligate anaerobic *Acidobacteria* in the tank bromeliad slurry would explain why they decreased at low moisture implying a elevated oxygen exposure.

Similarly to *Acidobacteria*, *Bacteroidetes,* mainly represented by *Sphingobacteriales,* also decreased with increasing drought. Oxygen was probably not the relvant stressor, since the *Sphingobacteriales* were mainly represented by *Mucilaginibacter* spp. which are so far known as aerobic or facultative anaerobic bacteria (Pankratov *et al*., 2007; Madhaiyan *et al*., 2010; Baik *et al*., 2010; Jian *et al*., 2012). *Mucilaginibacter* spp. can degrade pectin, xylan and laminarin (Pankratov *et al*., 2007). Therefore, the observation of *Mucilaginibacter* is consistent with a previous assumption that some tank slurry inhabiting bacteria are capable of degrading pectin (Goffredi *et al*., 2011b).

It has been reported that the relative abundance of *Acidobacteria* GP1 and *Bacteroidetes* was significantly correlated with differences in soil pH. There, *Acidobacteria* GP1 decreased with increasing pH, whereas *Bacteroidetes* increased (Lauber *et al*., 2009). Likewise, Goffredi et al. (2011b) observed that the bacterial community is greatly influenced by acid-base conditions in tank bromeliads. However, the pH of the tank slurries was quite similar under all the treatments (**Chapter 3**, Brandt *et al*., 2014). Therefore, the main controller of the bacterial community shifts in the present study seems to be moisture and in turn oxygen exposure, so that the *Acidobacteria* species and *Mucilaginibacter* spp. are more sensitive to drought in tank bromeliad slurry than the *Burkholderia* spp..

The canopies of tropical forests are among the most species-rich terrestrial habitats on Earth (Bassett 2003; Lowman 2004). Thereby, tank bromeliads, as aquatic-terrestrial

ecosystems, inhabit a high diversity of organisms (McCracken and Forstner, 2014). Tank-bromeliads even promote the diversity of aquatic food webs in neotropical forests (Brouard *et al*. 2012). However, periods of drought seem to have a dramatically influence on the bacterial diversity in tank bromeliad slurry. With increasing drought the number of OTUs decreased whereas the relative abundance of *Burkholderia* increased. This trend negatively affected the Simpson's Diversity Index which takes into account richness and evenness. The decrease of the bacterial diversity may have further implication for the food-web structure in tank bromeliads since the bacteria and other microorganisms degrade and utilize organic matter and which are then preyed by larger invertebrates (Maguire, 1971; Brouard *et al*., 2011).

In summary, restricted water availability affected the bacterial community composition as well as their diversity in tank bromeliad slurry. These changes may have further implications for the food-web structure and the cycling of carbon in tank bromeliads (e.g methanogenesis) since especially methanogens are dependent on usable substrates, which are provided by several bacterial guilds (LeMer and Roger, 2001).

Plant derived effects

Our experiments also revealed plant-derived effects on the microbial communities in the tank slurries. The bacterial community composition in the bromeliad tanks was different from that in nearby sampled soil. It was further different when the tank slurries were incubated outside of the bromeliad plants. Tank bromeliads actively take up water from their tank (Zotz and Thomas, 1999) and therefore reduce the water content of the slurry. Such uptake does not happen if tank slurries are incubated outside of bromeliad tanks. Therefore, one plant-derived effect on the bacterial community was the uptake of water from the tank slurry. The water level further influences the exposure to oxygen. In treatment C1 and C2 the tank slurry was covered with water during the whole incubation time presumably resulting in anoxic conditions and in anaerobic degradation of the organic matter. Treatment C3, on the other hand, had a similar water content as treatment P1 of the plant experiment and was therefore used for a direct comparison. It is well known that plants can stimulate or shape the surrounding microbial community by secreting deposits through their roots into the soil (Paterson *et al*., 2007, Wei *et al*., 2014). Analogously, tank bromeliads may secrete deposits into their tanks thus

influencing the inhabiting microbial communities. Indeed, the relative abundances of *Acidobacteria*, *Actinomycetales* and *Sphingobacteriales* were significantly higher in the plant environment. *Acidobacteria* are assumed to interact with plants or with plant-associated microbial communities (da Rocha *et al*., 201). Consistent to our study, Goffredi et al. (2011b) detected more clones of *Acidobacteria* in the tanks of Costa Rican bromeliads than in amber bottles, intended to artificially simulate a bromeliad tank in the field. In contrast, clones belonging to the *Sphingobacteriales* were more abundant in the artificial catchments (Goffredi *et al*., 2011b). However, a direct comparison to our study is problematic, since the tank substrates originated from different geographic sites and sampling and incubation conditions were different.

The main finding of this study is the dominant presence of the *Burkholderia* species regardless of the incubation conditions. Phylogenetic analysis showed a remarkable close clustering of the *Burkholderiales* sequences taken from treatment P3 and treatment C1 indicating the presence of the same species under drought conditions irrespectively of the influence of the plant. The order of *Burkholderiales* is described as quite versatile including aerobic and facultatively anaerobic organisms. Members of the *Burkholderia* seem further to be quite resistant against drought, since *Burkholderia pseudomallei* could be recovered from wet tropical soil after 91 days desiccation (Larsen *et al*., 2013).

In conclusion, the *Burkholderia* species showed an enhanced resistance to changing water levels, which can regularly occur under natural conditions in tank bromeliads depending on the tank capacity, the influence of evaporation (Zotz and Thomas, 1999), the location of the plant or on changes in precipitation patterns.

In summary, this is the first study investigating the effect of water on the bacterial community in tank bromeliad slurry incubated inside and outside of bromeliad tanks. The bacterial community was affected by the availability of water and the incubation environment. In our tank slurry we identified the *Burkholderia* as the most resistant bacterial group indicated by its dominant presence under all tested incubation conditions. We have further shown that bromeliad tank slurry is inhabited by microbes which are involved in CH_4 cycling (**Chapter 3**, Brandt *et al*., 2014) and in nitrogen cycling.

4.6 References

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5. Leaf axils of oil palms- a potential habitat for methanogenesis

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A status report

Contributions
FBB designed the study, performed the sampling, performed all lab work, evaluated all data, performed statistical analysis and wrote the manuscript
GOM designed the study, performed the sampling and wrote the manuscript
RC wrote the manuscript

5.1 Abstract

Several studies suggest the contribution of terrestrial vegetation, including phytotelmata (tank bromeliads, tree holes, pitcher plants, non-bromeliad leaf axils) to the global methane budget. Between the leaf axils of oil palms epiphytes are growing and dead organic material accumulates. In the present study, we investigated if these niches may provide a potential habitat for methanogenesis. Therefore, we examined the abundance and composition of the bacterial, archaeal and methanogenic community in the organic material of oil palm leaf axils, and assessed the potential for production of CH_4. The microbial community size was lower in material from leaf axils of oil palms than of tank bromeliads sampled in the vicinity. However, when the organic material was incubated under moist anoxic or water-logged oxic conditions, the size of the methanogenic community increased. Abundance of the *mcrA* marker gene increased from ~10^5 copies gdw^{-1} before to ~10^7 copies gdw^{-1} after the incubation of oil palm material. After 10 days of incubation, CH_4 concentrations started to increase reaching production rates of 510 ± 80 nmol gdw^{-1} d^{-1} and 450 ± 90 nmol gdw^{-1} d^{-1} in the anoxic and oxic treatments, respectively. In conclusion, leaf axils of oil palms seem to be a potential habitat for CH_4 formation.

5.2 Introduction

Palm oil is increasingly used for the production of processed foods and biodiesel (Reijnders & Huijbregts, 2008; Fitzherbert *et al*., 2008). Therefore, the oil palm (*Elaeis guineensis*) has become one of the most rapidly expanding equatorial crops during the last years. In fact, the cultivation of oil palms has increased from 3.6 million ha in 1961 to 13.2 million ha in 2006 (FAO, 2007) whereby Malaysia and Indonesia represent the two largest oil palm producing countries. The cultivation of oil palms has already considerable environmental impact, e.g., resulting in the loss of natural habitats (Fitzherbert *et al*., 2008) and in damaging the integrity of aquatic ecosystems (Cunha *et al*., 2014). The conversion of forests into oil palm plantations is mainly achieved by burning of the original vegetation, thereby causing greenhouse gas emission. Soils and plant biomass contain together ~2.7 times more carbon than the atmosphere, representing the largest biologically active stores of terrestrial carbon (Schlesinger, 1997). The carbon dioxide (CO_2) emission by forest conversion clearly exceeds the potential carbon fixation by oil palms (Germer and Sauerborn, 2007). Besides CO_2, there are in addition methane (CH_4), carbon monoxide (CO) and non-methane volatile hydrocarbons, which are released into the atmosphere by burning of the vegetation (Reijnders & Huijbregts, 2008). Therefore, to fulfill demands of Sustainability (Basiron, 2007), it is recommended that the expansion of oil palm plantation should be restricted to pre-existing cropland habitats (Koh and Wilcove, 2008).

However, studies concerning gas emission and the associated microbial communities from existing oil palm plantations are rare, and habitats on the oil palm trees have so far not been investigated. Mature oil palms are 20-30 m high. The early growth of a palm tree results in a stem base and after 3 years in the formation of a trunk, which grows every year by 25-50 cm. The leaves grow in a spiral way out of the meristem. The crown of the trees consists of 40-50 leaves, whereby a mature tree develops up to three leaves per months. The stem is covered by the bases of old leaves, which are regularly cut during crop harvesting or maintenance (Verheye, 2010). Between these leaf bases moss and other epiphytes grow and organic material accumulates (Ridley 1930, Figure 5.1). The leaf bases of oil palms form catchments that are similar to those of tank bromeliads. Tank bromeliads are phytotelmata in neotropical forests which collect organic matter and water (=tank slurry) between their leaf axils for their nutrient demand, and provide a

unique habitat for (microbial) organisms (Kitching, 2001; Pittl *et al*., 2010; **Chapter 2**). The water saturated conditions in tank bromeliads enable the anaerobic degradation of organic matter and the production of CH_4 (Martinson *et al*., 2010). Common acetoclastic and hydrogenotrophic methanogenic archaea were detected in Ecuadorian and Costa Rican tank bromeliad slurries (Martinson *et al*., 2010, Goffredi *et al*., 2011a). Their composition and the pathway of CH_4 formation are affected by water availability (**Chapter 3**, Brandt *et al*., 2014).

We hypothesized that the organic material between the leaf axils of oil palms may also provide a habitat for methanogenic microorganisms allowing CH_4 production. Therefore, we incubated the organic material sampled between the leaf axils of oil palms from a Cost Rican plantation under anoxic as well as under water-covered oxic conditions, and determined the structure of the bacterial, archaeal, and methanogenic communities together with the potential production of CH_4 and its stable carbon isotopic composition.

5.3 Material and methods

Sampling site and incubation

The field station La Gamba, situated in the southwest of Costa Rica on the edge of the National Park Piedras Blancas (N 8°42"61', W 83°12'97") is one of the last pristine rainforests in the lowlands at the Pacific coast of Central America. The climate in this region is characterized by high rainfall (6.000 mm y^{-1}, Hofhansl *et al*., 2014). Adjacent to the tropical station, there is a 6-ha large oil palm plantation. The palm trees were 8 years old and had a height of 10 m. Organic material, located between the leaf axils of oil palm trees were sampled in May 2012 from 3 leaf axils (up to 2 m height) using 6 randomly chosen trees. The material was immediately transported in cooled state (4°C) to the Max-Planck-Institute for Terrestrial Microbiology in Marburg, Germany.

The material was pooled and 3 x 0.3 g were taken as reference samples (T_0) for molecular analyses. For incubation, 12 pressure tubes (27 ml) were filled with 3 g (fresh weight) organic material and closed with rubber stoppers and aluminum caps. In 6 of the tubes 3 ml sterile H_2O-dest was filled. These tubes were incubated under oxic condition and therefore flushed with synthetic air (21% O_2, 79% N_2) for 5 min (treatment OX). Into 3 of these tubes methyl fluoride (CH_3F) was filled (treatment OXM) to a final concentration of 2% to inhibit acetoclastic methanogenesis (Janssen and Frenzel 1997). Oxygen concentration was measured every 2-3 days and if required, the air headspace was replaced to maintain ambient atmospheric conditions (see Figure S5.1a). The other 6 pressure tubes were incubated under anoxic conditions without additional water and therefore, flushed with N_2 for 5 min (treatment A). In 3 of these tubes CH_3F was added (treatment AM). All samples were incubated in the dark at 25°C over 50 days.

Gas samples of 250 µl were regularly taken from the headspace using a gas-tight pressure lock syringe (Vici, Baton Rouge, LA, USA) and analyzed immediately. Methane and CO_2 concentrations were analyzed using a gas chromatograph (Figure S5.1b, c) equipped with a methanizer (Ni-catalyst at 350°C, Chrompack, Varian Deutschland GmbH, Darmstadt, Germany) and a flame ionization detector (Shimadzu Deutschland, Duisburg, Germany). Gas production rates were calculated from the data obtained between days 28 to 34. Ratios of carbon isotopes (^{13}C:^{12}C) in CH_4 and CO_2 were measured using a gas chromatograph combustion isotope ratio mass spectrometer as previously described by

Conrad et al. (2009). Apparent enrichment factors (ε) were calculated as described in Brandt et al. (2014).

Therefore, the fractionation factor (α) was calculated by:

$$\alpha = (\delta_{CO2}+1000)/ (\delta_{CH4}+1000) \quad \text{(a)}.$$

δ_{CO2} and δ_{CH4} are the isotopic signatures of the carbon in the CO_2 and CH_4. The apparent enrichment factor ε_{app} (in ‰) was determined through:

$$\varepsilon_{app} = 1-\alpha \quad \text{(b)}.$$

The pH of the organic substrate was directly measured in the field and after the incubation of 50 days. Further, total carbon and nitrogen content was determined on a CHNS-elemental analyzer by the Analytical Chemical Laboratory (University of Marburg). Gravimetric water content was measured by drying 3×1 g oil palm substrate at 65°C for 72 h.

Acetate concentration in the organic substrate was determined before and after the incubation. Prior to measurement, the organic matter was mixed with distilled water at a ratio of 1:2, and shaken for 1.5 h at 25°C. The supernatant was sampled with a sterile syringe, membrane-filtered (0.2 µm) and stored frozen until analysis of acetate concentration using high performance liquid chromatography (Sykam, Gilching, Germany) with refractive index and UV detectors. Acetate concentration in the supernatant was determined using an external standard solution containing 10 mM acetate (Sigma-Aldrich).

Molecular analysis

After the incubation the pressure tubes were opened and 0.3 g organic material was taken from each tube for molecular analysis. DNA was extracted from the T_0 and incubation samples using the Machery and Nagel NucleoSpin® Kit for Soil following the manufacturers' instruction. At the end DNA was dissolved in 50 µl of nuclease free water. DNA was checked for quality and quantity by gel electrophoresis and using a ND1000 spectrophotometer (NanoDrop). Quantification of microbial groups was performed using quantitative PCR (qPCR) targeting the bacterial and archaeal 16S rDNA and the methanogenic marker gene *mcrA* using the primer combination Ba519f/Ba907r (Stubner,

2002), Ar364f/Ar934br (Burggraf *et al*., 1997/Grosskopf *et al*., 1998) and MCRf/MCRr (Springer *et al*., 1995), respectively. PCR reactions were conducted in 96-well plates in an iCycler thermocycler equipped with the BioRad IQ 2.0 Standard Edition Optical System software (BioRad) using a SYBR green I assay. Each qPCR reaction contained in a total volume of 25 µl, 1x SYBR®Green Ready Mix™ (Sigma), 3 mM $MgCl_2$ (Sigma), 0.6 µM of each primer and 1 µM FITC (fluorescein isothiocyanate; BioRad) as well as 2 µl template DNA. For all qPCR assays, standards containing known numbers of DNA copies of the target gene were used. The standard was applied in a dilution series containing 10^7 until 10^1 copies. Technical replicates of templates were done using 1:10 and 1:100 dilutions of the template DNA. Thermoprofiles for the quantification of the archaeal 16S rDNA and *mcrA* gene copies are described by Angel et al. (2011). The quantification of the bacterial 16S rDNA was done using the following thermoprofile: initial denaturation 94°C for 8 then 45 cycles of 94°C for 20 sec, 50°C for 20 sec, 72°C for 50 sec. The purity of PCR products was checked by melting curves. Microbial community compositions were determined using terminal restriction fragment length-polymorphism (TRFLP) targeting the bacterial and archaeal 16S rDNA and the methanotrophic marker gene *mcrA*. The following primer combinations were used: 27f/907r for bacteria (Osborne *et al*., 2005; Muyzer *et al*., 1995) with a labeled 6-carboxyfluorescein (FAM) forward primer; 109f/934r for archaea (Grosskopf *et al*., 1998) with FAM-labeled reverse primer and MCRf/MCRr (Springer *et al*., 1995) for methanogens with FAM- labeled forward primer. PCR reactions were carried out in a total volume of 50 µl, containing 200 µM deoxynucleoside triphosphates (Fermentas), 1x GoFlexiGreen Buffer (Sigma-Aldrich), 10 µg Bovine Serum Albumin (Roche), 1 Unit GoTaq DNA Polymerase (Sigma-Aldrich), 0.5 µM of each primer pair, 1.5 mM $MgCl_2$ (Promega) and 20 ng template DNA. The PCR reaction was performed at 94°C for 3 min followed by 22 cycles for bacterial and 30 cycles for archaeal and *mcrA* gene amplification at 94°C for 30 s, 52 °C for 45 s, 72°C for 90 s and a final elongation step at 72°C for 5 min. PCR products were purified with the GenElute™ PCR Clean-Up Kit (Sigma) according to manufactures protocol. The purified amplicons of bacterial 16S rDNA were digested using *MspI* enzyme (restriction side: 5'-CCGG-3`, 37° C; 12 h; Fermentas). *TaqI* enzyme (restriction side: 5'-TCGA-3`, 65° C; 3 h; Fermentas) was used to digest archaeal 16S rDNA amplicons and *mcrA* gene amplicons were digested using *Sau96I* FastDigest® (Fermentas) for 15 min at 36°C. The purification of the fragmented DNA was performed

using SigmaSpin™ post-reaction clean-up columns (Sigma-Aldrich) following manufactures' protocol. The capillary fragment size separation was conducted with an ABI PRISM 3130 Genetic Analyzer (Applera Deutschland GmbH). The TRFLP data were obtainend using the software Genescan 4.0 (Applied Biosystems) by comparison with an internal DNA standard. The resulting TRFLP profiles were standardized as described in Dunbar et al. (2001) using the peak area (Figure S5.2).

Statistical analysis

The statistical analyses were conducted in R version 2.10.1. Differences in gene copy number and single TRF abundances between incubation treatments were determined using the one-way analysis of variance (ANOVA) and the *post hoc* Tukey (HSD) test. All data were checked for normality and homoscedasticity using Kolmogorov–Smirnov and Levene's test, respectively. Differences in community compositions, based on TRFLP profiles were analyzed using *permutational* multivariate analysis of variance (Permanova). All levels of significance were defined at $P \leq 0.05$. ANOVA were done using the stats package. Permanova were done using package vegan.

5.4 Results

Figure 5.1 Oil palm plantation near the tropical field station La Gamba in the south-west of Costa Rica (a). Epiphytes and organic material is located between the leaf axils of oil palms (b, c).

Organic material from leaf axils were investigated in original state (T_0) and after incubation. The incubations were done with the material in original moist state under an anoxic gas phase (A) as well as under water-logged conditions and an oxic gas phase (OX). With both treatments, the creation of anoxic habitats inside the material, potentially with active methanogenesis, should be stimulated. Both treatments were done with and without CH_3F (M), an inhibitor of acetoclastic methanogenesis.

The oil palm material (T_0) had a gravimetric water content of 35% and was acidic with a pH of 4.8 ± 0.1. After the incubation the pH was 5.3 ± 0.1 in the oxic incubations (OX), pH 5.2 ± 0.1 in the oxic incubations plus CH_3F (OXM), pH 5.0 ± 0.1 in the anoxic incubations

(A) and pH 4.9 ± 0.1 in the anoxic incubations plus CH_3F (AM). The organic matter had a high total organic carbon content of 38 ± 1.3% and a nitrogen content of 2.2 ± 0.1%. Acetate concentration was in treatment OX and in one replicate of treatment AM not detectable. In the T0 samples acetate concentration was 0.5 ± 0.05 mM, in treatments OXM 1.8 ± 0.4 mM, in treatments A 2.3 ± 0.25 mM and in treatments AM 7.7 ± 2.6 mM.

Bacterial 16S rDNA copy numbers were similar in all the original and incubated samples and ranged between 3.5×10^9 and 5.6×10^9 copies gdw^{-1} (Figure 5.2a). Archaeal 16S rDNA copies were two orders of magnitudes lower (Figure 5.2b). Gene copies of the methanogenic marker gene *mcrA* were low in the original samples, but significantly increased after incubation. Incubations with CH_3F showed up to one order of magnitude lower *mcrA* gene copy numbers than incubations without CH_3F (Figure 5.2c).

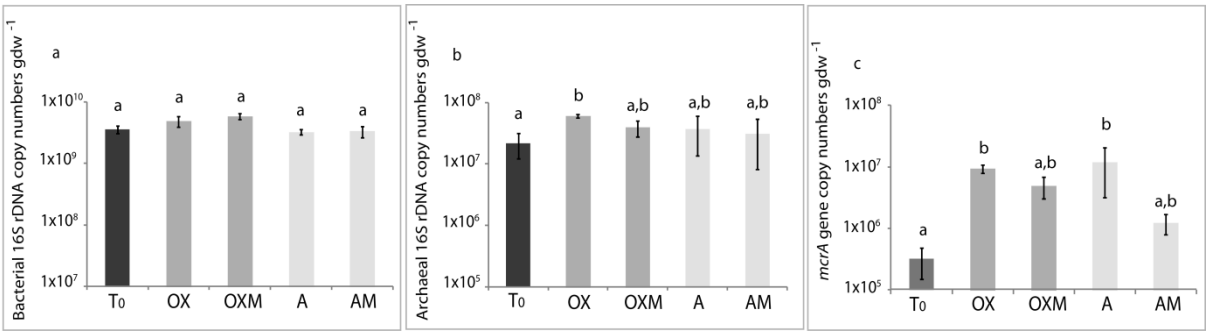

Figure 5.2 Bacterial (a) and archaeal (b) 16S rDNA and *mcrA* gene (c) copy numbers in organic material sampled from the leaf axils of oil palms. T_0 represents the reference sample before incubation. The other columns represent copy numbers after the incubation under water-logged oxic conditions without (OX) and with 2% CH_3F (OXM), and under moist anoxic conditions without (A) and with CH_3F (AM). Error bars represent standard deviations, n=3. Different letters indicate significant differences ($P \leq 0.05$).

The T-RFLP patterns of the bacterial, archaeal and methanogenic communities changed after the incubation in comparison to the T_0 sample, but statistically significantly only for anoxic conditions (A/AM) (Figure 5.3). The bacterial community was generally dominated by three TRFs of 137, 143 and 148 bp (Figure 5.3a). The relative abundance of the 265-bp TRF significantly increased during the incubation in comparison to T_0 and was more prominent under oxic than anoxic conditions. However, the 453-bp TRF significantly increased in the anoxic incubations. The archaeal community was dominated by a 186-bp TRF in all samples (Figure 5.3b). In comparison to the T_0 samples the 258-bp TRF significantly decreased during the incubations and almost disappeared under oxic conditions. The 382- and 393-bp TRFs were predominantly present under oxic conditions

and were lower in their relative abundance under anoxic conditions. The methanogenic community, characterized by the *mcrA* marker gene, was dominated by a 234-bp TRF in the T_0 samples and under oxic condition whereas the 391-bp and 423-bp TRFs were predominantly present under anoxic conditions.

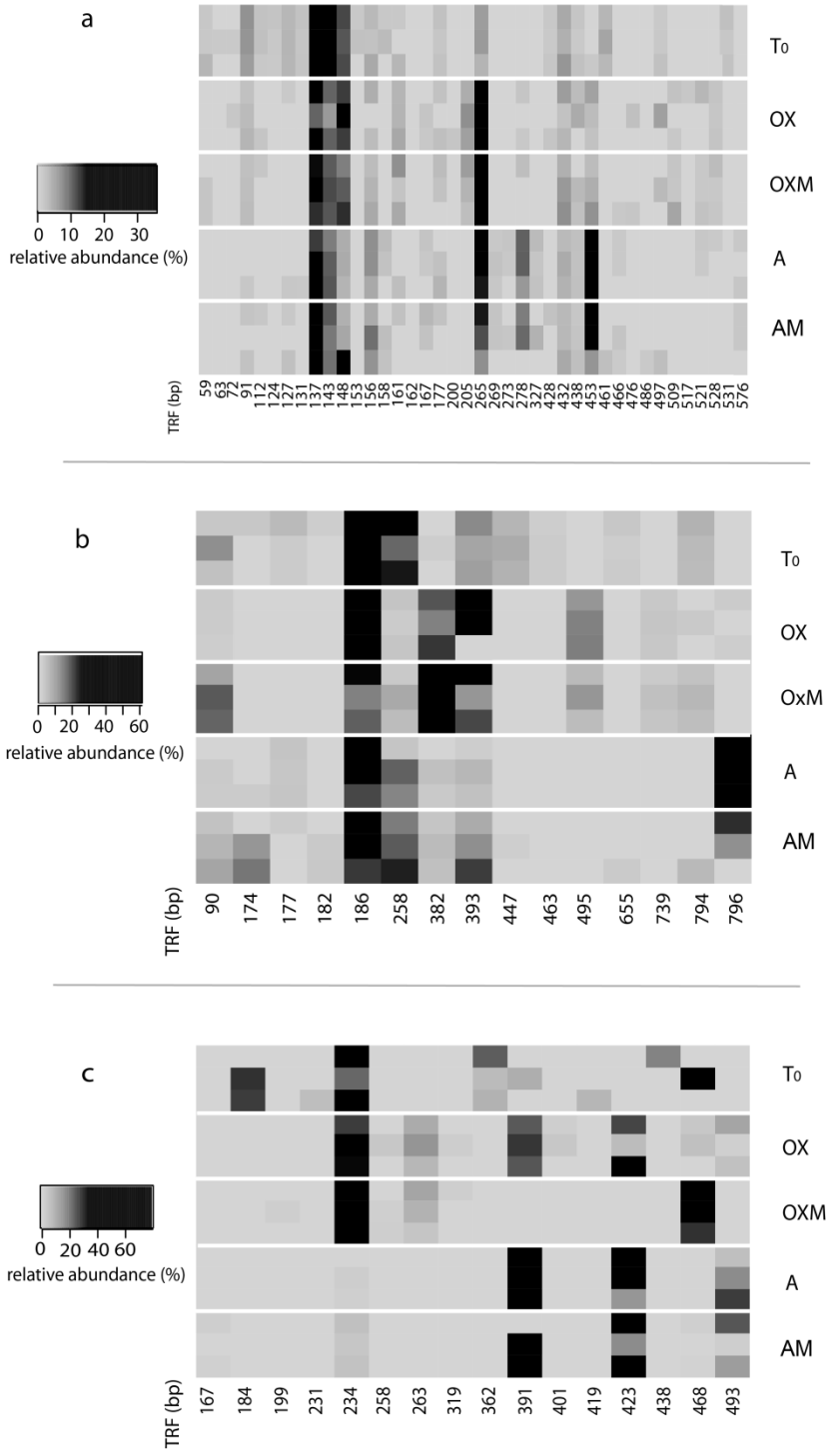

Figure 5.3 Heatmap of the bacterial (a), archaeal (b) and methanogenic (c) community based on TRFLP profiles targeting the bacterial and archaeal 16S rDNA and the methanogenic marker gene *mcrA*, respectively, in material sampled from oil palm leaf axils. Community profiles were obtained prior to the incubation (T_0) and after the incubation under water-logged oxic without (OX) and with CH_3F (OXM), and under moist anoxic conditions without (A) and with CH_3F (AM). Heat map colors represent relative abundance (%) of the TRFs.

Gas measurement in the incubations showed that concentrations of CH_4 started to increase after 10 days (Figure S5.1b). Methane concentrations increased in all flasks except one replicate from treatment AM, in which also acetate was not detectable. Therefore, calculation of emission rates and enrichments factors were made with only two replicates for treatment AM and statistical analyses were not performed. However, the CH_4 production rate was four times higher in treatment OX than in treatment OXM and two times higher in treatment A than in treatment AM (Figure 5.4a). Production rates of CO_2 were higher in the water-logged oxic than in the moist anoxic incubations (Figure 5.4b). The isotopic signatures ($\delta^{13}C$) of the CH_4 and CO_2 are shown in Figure 5.5. The $\delta^{13}C$-CO_2 values ranged during the incubation between -23‰ to -26‰, slightly lower in the anoxic incubations, and were not much affected by the addition of CH_3F (Figure 5.5b, d). The $\delta^{13}C$-CH_4 values in the oxic incubations clearly differed between the treatments OX and OXM (Figure 5.5a). With the addition of CH_3F and the inhibition of the acetoclastic methanogenesis the $\delta^{13}C$-CH_4 values decreased. The same trend was observed for the anoxic incubation but not as pronounced as under oxic conditions (Figure 5.5a, c). The CH_4 produced in the anoxic incubations had relatively high $\delta^{13}C$-CH_4 values resulting in less negative enrichment factors (Table 5.1).The enrichment factor showed throughout lower values in the oxic (OX, OXM) than in the anoxic treatments (A, AM), with or without the addition of CH_3F (Table 5.1).

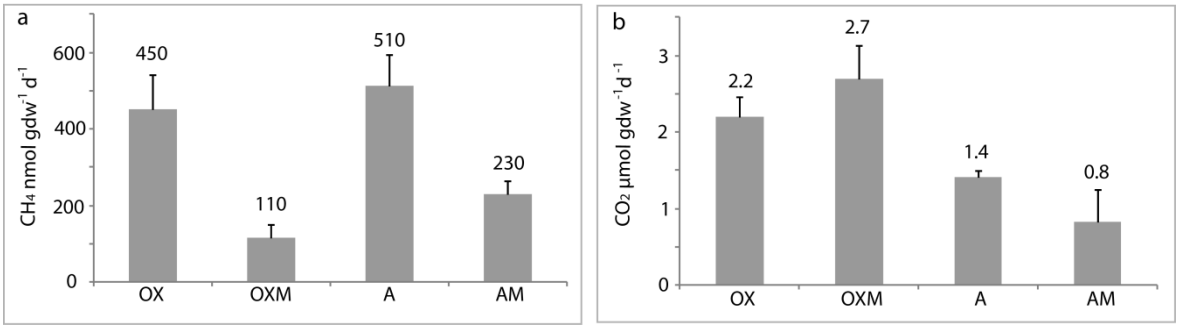

Figure 5.4 CH_4 (a) and CO_2 (b) production rates in oil palm organic material incubated under water-logged oxic (OX, OXM) and moist anoxic conditions (A, AM). M indicates the presence of 2% CH_3F. Production rates were calculated between day 28 and 34. Error bars represent standard deviation (n=3) except for CH_4 production rate in treatment AM (n=2).

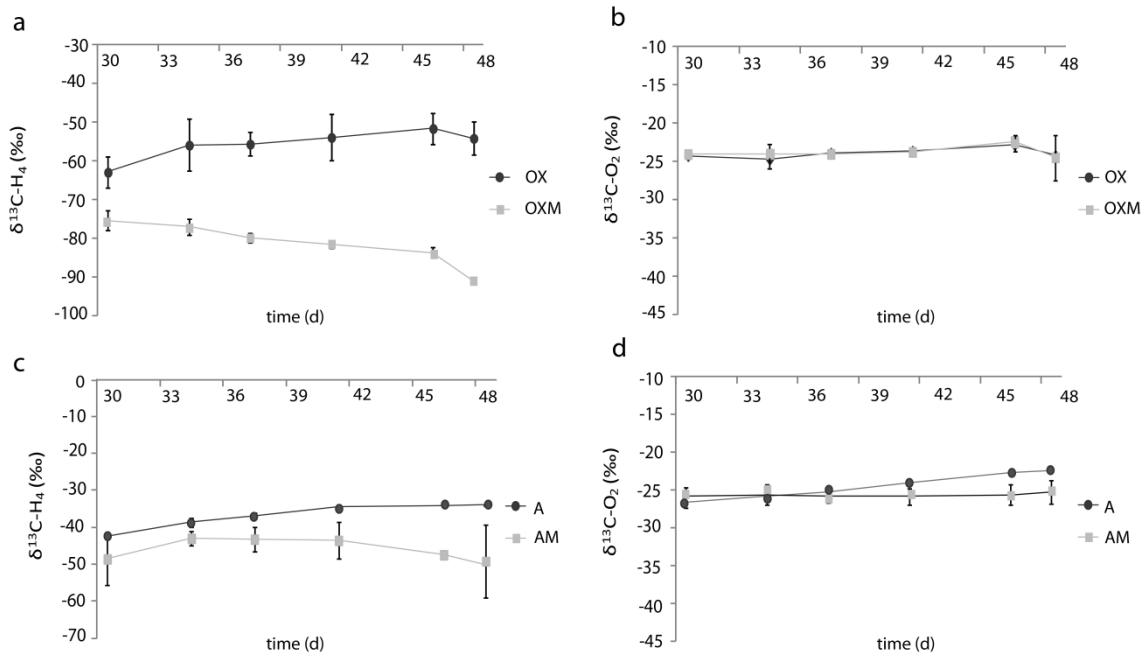

Figure 5.5 Isotope values for CH_4 (a, c) and CO_2 (b, d) during the incubation of oil palm material under water-logged oxic (OX, OXM) and moist anoxic conditions (A, AM) without (OX, A) and with (OXM, AM) CH_3F. Error bars represent standard deviation (n=3) except for $\delta^{13}C$-H_4 in treatment AM (n=2).

Table 5.1 Effect of different incubation treatments (OX= oxic with water, OXM= oxic with water and CH_3F, A= anoxic, AM= anoxic with CH_3F) on the apparent enrichment factor ε (‰) of ^{13}C in CH_4 compared to CO_2. Values represent the mean ± standard deviation of three replicates except for treatment AM (n=2).

day	30	34	37	41	46	48
OX	-41.3± 7.5	-33.1±4.9	-33.8± 6.4	-32.1± 4.3	-30.4± 5.9	-31.8± 2.6
OXM	-57.6±2.3	-60.9±1.8	-62.8±1.3	-65.6±1.2	-75.9±1.0	-73.9±4.0
A	-16.4±0.9	-13.4±1.1	-12.2±1.1	-10.9±0.9	-12.1±0.1	-11.8±0.2
AM	-23.5±8.3	-17.4±2.3	-17.8±3.8	-17.9±5.5	-22.4±4.8	-25.7±9.4

5.5 Discussion

Our results identified the leaf axil material of oil palms as a potential habitat for microbial CH_4 production. This was indicated by the presence of reasonably large number of methanogenic archaea, which further increased upon incubation under either anoxic or water-logged conditions, and by the eventual production of CH_4 under these incubation conditions.

The organic matter in the leaf axils of oil palms had a high carbon content and a slightly acidic pH, similarly as observed for leaf axils of tank bromeliads (Goffredi *et al*., 2011a; **Chapter 3**, Brandt *et al*., 2014). The main carbon compound in the leaf axil environment of tank bromeliads is plant-derived cellulose, which is assumed to be often incompletely degraded unless degradation operates over several months (Goffredi *et al*., 2011a). The low pH values found in tank bromeliads are suggested to be the result of the plants pumping H^+ into the tank water in exchange for nutritional cations (Lopez *et al*., 2009). However, such cationic exchange is probably not the explanation for oil palms, since these trees take up their nutrients via their root system (Corley and Tinker, 2008). Nevertheless, the low pH in the leaf axil environments is probably a major determinant for microbial colonization and community composition (Lopez *et al*., 2009), as it was shown for tank bromeliads (**Chapter 2**; Goffredi *et al*., 2011b). The potential for CH_4 formation in the material from oil palm leaf axils was indicated by the presence of a methanogenic archaeal community of ~10^5 cells per gram dry weight (actually 10^5 *mcrA* gene copies gdw^{-1}). These numbers of methanogens were 2-3 magnitudes lower in comparison to those in tank bromeliad slurry, collected at the same sampling site (**Chapter 2**) or to flooded rice field soils (Watanabe *et al*., 2009, Björn Breidenbach, personal communication), a typical place for CH_4 formation. However, the oil palm material did not produce CH_4 in its original state, but started to emit CH_4 after a lag – phase of 10 days probably due to the previous proliferation of methanogenic archaea as indicated by their increase to ~10^7 *mcrA* gene copies gdw^{-1} during the incubation. A similar behavior was observed in biological soil crusts from desert soil samples. Angel et al. (2011) quantified ~$5x10^4$ *mcrA* gene copy numbers gdw^{-1} *in situ*. Then, upon incubation with water almost all samples formed CH_4 after about one week in parallel with proliferation of the methanogens (Angel *et al*., 2011). Equally to this study, CH_4

concentration increased during incubations under either moist anoxic or water-logged oxic conditions without the addition of further substrates.

In the organic material from oil palm leaf axils the potential rates of CH_4 formation were as high as 510 nmol gdw^{-1} d^{-1}. In comparison, tank slurry substrates of Ecuadorian bromeliads showed lower production rates ranging between 10 and 15 nmol CH_4 gdw^{-1} d^{-1}. Contrary to the present study, the bromeliad tank substrates were incubated in moist state but neither water-logged nor under anoxic conditions (**Chapter 3**, Brandt *et al*., 2014). Nevertheless, both studies indicate that the organic material from leaf axils of phytotelmata have the potential to produce CH_4 under ambient conditions when the material is sufficiently wet to allow the creation of anoxic niches. These conditions may occur on a regular basis, since oil palms or tank bromeliads occur, contrary to deserts, at sites that are characterized by a high annual precipitation (6000 mm^{-1}; Hofhansl *et al*., 2014). The occurrence of anaerobic niches is also indicated by the presence of denitrifying communities in leaf axil material from Indonesian oil palms, in which N_2O production starts immediately upon anoxic incubation (Suleiman *et al*., in preparation). The degradation of organic matter is achieved by the sequential reduction of O_2, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-} and CO_2, and methanogenesis takes place as the final step in highly reduced environments (Ponamperuma, 1972; Patrick and Reddy, 1978). According to the thermodynamic theory electron acceptors with a higher redox potential will be reduced first (Zehnder and Stumm, 1988). This sequential reduction may explain the lag-phase until methanogenesis starts, since at least denitrification, using NO_3^- as electron acceptor, seem to play a role in oil palm material. In conclusion, these results indicate that leaf axils of oil palms inhabit microbial communities that are potentially involved in carbon and nitrogen cycling under anoxic conditions.

Microbial community composition changed under different incubation conditions. The identification of inhabiting microbial species, potentially involved in carbon cycling, has to be conducted in future studies. However, for the archaeal community composition in oil palm substrate we can make few assumptions since we were already able to assign several TRFs to specific archaeal lineages in tank bromeliad slurry (**Chapter 3**, Brandt *et al*., 2014).

In the present study, the archaeal community was generally dominated by a 186-bp TRF. This TRF has previously been assigned to Crenarchaeota or *Methansosarcinaea* (**Chapter 3**, Brandt *et al*., 2014). Under water-logged oxic conditions the relative abundance of the 382-bp TRF and the 393-bp TRF increased, which were assigned to *Thermoplasmatales* and *Methanomicrobiales*, respectively, in tank bromeliad slurry (**Chapter 3**, Brandt *et al*., 2014). Differences in the microbial community composition may explain the different isotopic signatures of produced CH_4 under anoxic or water-logged oxic conditions. The isotopic signature of CH_4 can vary according to different pathways of methanogenesis. During hydrogenotrophic methanogenesis the isotopically lighter carbon is strongly preferred, whereas the isotope effect is less expressed when the methyl group of acetate is used for CH_4 production (de Graaf *et al*., 1996; Weimer and Zeikus, 1998). For instance, the dominance of the 393-bp TRF, potentially representing hydrogenotrophic *Methanomicrobiales*, may explain the relatively low isotopic signatures of CH_4 in treatment OX/OXM, since *Methanomicrobiales* prefer the isotopically lighter carbon CO_2 for CH_4 production. A less negative enrichments factor, as it is observed under anoxic conditions, is typical for the consumption of acetate. Members of *Methanosarcinaceae*, potentially represented by the 186-bp TRF, are able to perform both acetoclastic and hydrogenotrophic methanogenesis (Liu and Whitman, 2008). For instance, the performance of acetoclastic methanogenesis in *Methanosarcina barkeri* resulted in an enrichment factor around -27‰ (Goevert and Conrad, 2009).

However, the enrichment factors determined under different incubation conditions gave a complex picture. In general, the enrichment factors under moist anoxic incubation conditions were less negative than in water-logged oxic conditions indicating the dominance of different methanogenic pathways under these conditions. The more negative enrichment factor under water-logged oxic conditions indicates dominance by hydrogenotrophic methanogenesis, but is contrasted by a strong effect of CH_3F, which is characteristic for the operation of acetoclastic methanogenesis. The presence of acetoclastic methanogens is also indicated by an accumulation of acetate in treatments with CH_3F which inhibits the acetoclastic methanogenesis. The relatively low fractionation under moist anoxic conditions could be caused by a dominance of acetoclastic methanogenesis. This assumption is also strengthened by an increase of acetate with the addition of CH_3F. However, the relatively small effect of CH_3F is contradictory.

In conclusion, this is the first study investigating the microbial community in substrate of oil palm leaf axils. The microbial community size was lower as in comparable sites like tank bromeliad leaf axils. However, the methanogenic community as well as CH_4 formation increased during anoxic or water-covered oxic conditions. Therefore, our results strengthen the assumption that leaf axils of oil palms seem to be a potential niche for CH_4 formation. Nevertheless, our results have to be verified in a comprehensive sampling of oil palm substrate, investigating single trees and different sites accompanied with *in situ* gas measurements. Further, the identification of inhabiting microbial species, potentially involved in carbon cycling, has to be conducted.

5.6 References

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5.7 Supplementary material

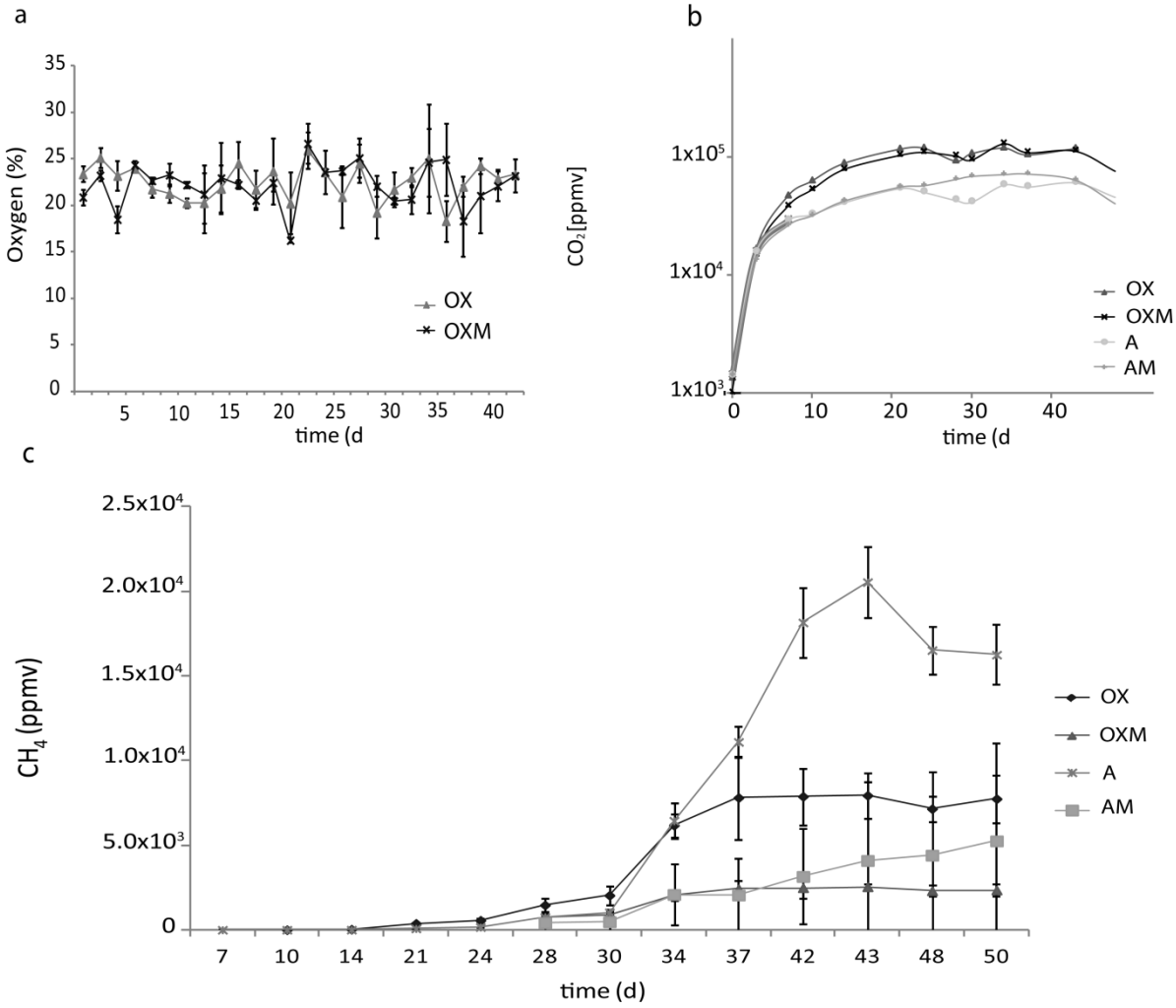

Figure S5.1 Oxygen (a), CO_2 (b) and CH_4 (c) concentration in the headspace of incubation flasks with organic matter sampled between the leaf bases of oil palms under oxic conditions with water (OX), under oxic conditions with water and CH_3F (OXM), under anoxic conditions (A) and under anoxic conditions with CH_3F (AM). Values represent the mean ± standard deviation of three replicates except for treatment AM (n=2).

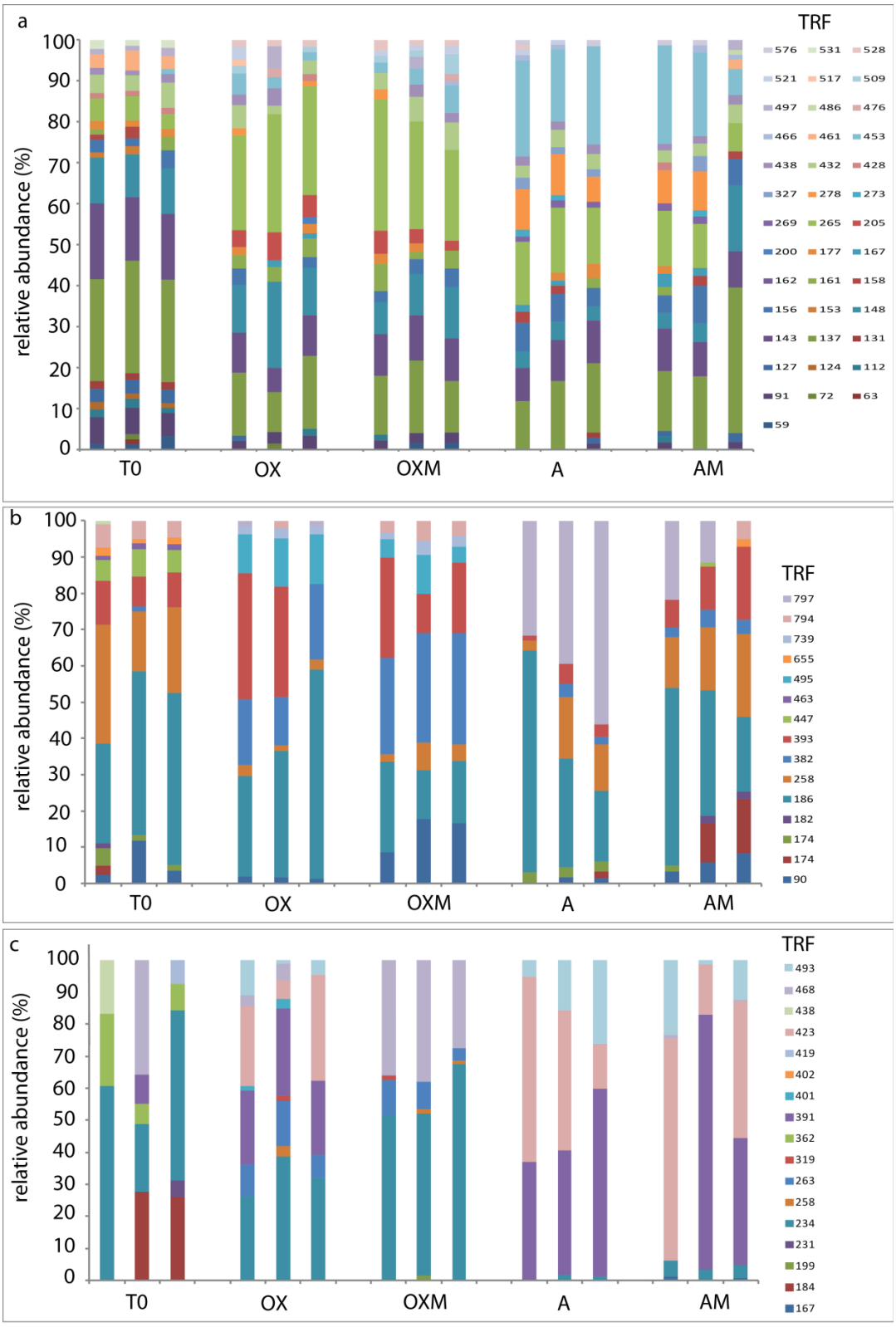

Figure S5.2 TRFLP profile of the bacterial (a), archaeal (b) and methanogenic (c) community in oil palm substrate before (T0) and after the incubation under oxic conditions with water (OX) under oxic conditions with water and CH_3F (OXM), under anoxic conditions (A) and under anoxic conditions with CH_3F (AM). The graph shows the relative abundances of TRFs as a measure of the community composition by targeting the bacterial and archaeal 16S rDNA and the methanogenic marker gene *mcrA*.

6. General discussion and concluding remarks

Beside permanent flooded wetlands there exist distinct wetlands created by phytotelmata including the catchments of pitcher plants, bamboo nodes, tree holes, tank bromeliads and non-tank-bromeliad leaf axils. Phytotelmata possibly make a larger contribution to the global methane (CH_4) budget than previously assumed. Tank bromeliads were identified to emit substantial amounts of CH_4 into the atmosphere over neotropical forests. Many different bacteria and archaea, which are commonly found in soils, were also detected in tank bromeliads. Nevertheless, little is known about the methanogenic microbial communities in tank bromeliads and the factors that influence their activity.

Therefore, this thesis explored (i) the methanogenic microbial community colonizing leaf axil environments of tank bromeliads and (ii) factors that influence these communities in composition, abundance and activity **(Chapter 2, 3, 4)**. The microbial community composition and abundance was assessed using several molecular techniques (T-RFLP, 454 pyrosequencing, qPCR) targeting the bacterial and archaeal ribosomal 16S rRNA and 16S rRNA gene and the methanogenic and methanotropic marker gene *mcrA* and *pmoA*, respectively. The microbial activity was investigated by gas measurements and the methanogenic pathway was determined by isotope measurements of produced CH_4. In order to study factors that influence the tank inhabiting methanogenic communities, the tank bromeliad was established as a model system in the greenhouse. Therefore, this thesis comprises data from field (Costs Rica; **Chapter 2**) and laboratory (**Chapter 3, 4**) experiments.

In the second part of the thesis we explored a further potential site for methanogenesis in a leaf axil environment. This is the first study identifying the leaf axils of oil palms as a potential site for methanogenesis (**Chapter 5**). The abundance and community composition was determined in the organic matter of oil palm leaf axils *in situ* samples as well as after incubation experiments, using T-RFLP and qPCR. The potential of methanogenesis was investigated by gas measurements and the carbon isotopic signatures of CH_4 formed to determine the methanogenic pathway.

6.1 The tank bromeliad as a model ecosystem

The use of model organisms in biology (e.g. mice, fruit fly *Drosophila melanogaster*, bacterium *Escherichia coli*) is an effective and successful way to test hypotheses and to analyze defined processes. Specific questions can be directly addressed to model systems and their (often) short life times and small sizes allow frequent replications. Nevertheless, focusing on model systems will also lead to certain biases since natural processes will be dramatically simplified and may do not adequately reflect natural conditions (Bolker, 1994). Nevertheless, model systems allow us to gain the knowledge that can be then used to develop appropriate experiments of non-model systems. Especially ecologists, investigating complex field systems often face the problem to distinguish between interactions of environmental factors that are often related in diffuse ways (Jessup *et al*., 2004). Until today ecology has only a few model systems (e.g. Tribolium beetle for population ecology, Darwin finches for evolutionary ecology) but studies using model systems have significantly advanced the knowledge to understand processes that are too difficult to study in field systems (Jessup *et al*., 2004). Natural microcosms may provide a way to combine the advantage of tractability along with avoiding the usage of artificial systems. Natural microcosms are distinct habitats, containing <1 L in volume, such as pitcher plants, tree holes or tank bromeliads and provide more realistic field conditions than artificial microcosms (Srivastava *et al*., 2004). Nevertheless, microcosms may be also more sensitive to environmental influences than large systems do: Freezing, drought or litter fall can have dramatic effects on the inhabiting communities which may have minor consequences in a larger system. Nevertheless, the distinct habitats of microcosms may reduce the main problem of ecological studies: the precise delineation of communities (Srivastava *et al*., 2004). Therefore, tank bromeliads were frequently used to study food web structures (Kitching *et al*., 2001; Srivastava, 2006; Srivastava *et al*., 2008; Brouard *et al*., 2011), animal richness (Richardson, 1999), activity and distribution of (aquatic) invertebrates (Carrias *et al*., 2001; Marino *et al*., 2013) and microorganisms (Carmo *et al*., 2014). Further, manipulation of tank inhabiting organisms can be easily done and therefore, we established the tank bromeliad as a model system in the greenhouse to study microbial species that are involved in CH_4 cycling and plant-microbes interactions. In cooperation

with the breeding company 'Corn. Bak B.V.' (Netherlands) we successfully cultivate several bromeliad species in the greenhouse.

Using the tank bromeliad as a model system, allows us to investigate the methanogenic community under defined conditions and the ability to manipulate environmental influences such as water availability (**Chapter 3, 4**). Further, the potential influence of the plant on the inhabiting microorganisms can be studied (**Chapter 4**).

The tank organic slurry in this study originated from Ecuadorian and Costa Rican tank bromeliads, which were either directly analyzed (field data) or used for greenhouse or incubation experiments (laboratory data). The combination of field and laboratory data provided new insights into the microbial communities and CH_4 cycling in bromeliad tanks.

6.2 Microbial communities and methane cycling in tank bromeliads

One objective of this study was to shed light on the microbial communities in methane-emitting tank bromeliads. In **Chapter 2** we have shown that tank bromeliads provide a unique habitat for microorganisms that are involved in CH_4 cycling. The presence of methanogens and methanotrophs in all investigated tank slurries indicate the potential for both CH_4 formation and CH_4 oxidation in bromeliad tanks. Microbial communities were different to nearby sampled soil and differed between individual plants. Tank slurry properties like pH, carbon, nitrogen concentration and oxygen concentration were different between plants and affected microbial community composition. Tank slurry properties depend on the receipt of nutrients and so we assume that the place where a tank bromeliad develops may play a significant role in shaping the inhabiting microbial communities. Nevertheless, the explained microbial variation due to measured variables in this study was relatively low. This may be due to factors that were not measured but were of great importance, such as drying history which may influence microbial community composition. Hence, we investigated the effect of drying in **Chapter 3**. Although tank bromeliads can effectively store rainwater between their leaf axils, for the neotropics it is anticipated that drought periods will increase due to climate change (Cox *et al*., 2008; Malhi *et al*., 2008, Salazar *et al*., 2007). Kotowska and Werner (2013) showed that drought negatively affected CH_4 efflux in Ecuadorian tank bromeliads. In the present study we confirmed these results and further showed the impact of

moisture on the microbial communities inside bromeliad tanks. Thereby, we observed that the different water availabilities resulted in a shift from a hydrogenotrophic (*Methanobacteriales*) dominated to an aceticlastic (*Methanosaetaceae*) dominated methanogenic community. This trend was also seen in the isotopic signatures of the produced CH_4 revealing that hydrogenotrophically derived CH_4 dominated under high moisture. *Methanosaetaceae* were significantly increasing upon drought and oxygen exposure and seem therefore to be more oxygen tolerant than previously assumed.

Wu and Conrad (2001) assumed that *Methanosaeta* populations in rice field soil were better adapted to aeration stress, since resident populations recovered faster than *Methanosarcina* populations. At this time a possible explanation was not provided. With increasing knowledge due to genome sequencing analysis it was revealed that *Methanosarcina* spp. and *Methanocella arvoryzae* MRE50, described as strict anaerobes, also posses genes encoding for oxygen detoxifying enzymes (Erkel 2006; Figure 6.1a). Oxygen detoxifying enzymes can suppress the reactivity of reactive oxygen species, like hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\cdot -}$), which can highly damage cell components such as lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrates and nucleic acids (Cabiscol *et al*., 2010). Also the genome of *Methanosaeta concilii* GP-6 (Barber *et al*., 2011; NCBI Reference Sequence: NC_015416.1) possesses genes for antoxidant enzymes. A schematic presentation of the predicted enzymatic reactions is provided in Figure 6.1b. In general, cytoplasmic superoxide anions can be detoxified by two different types of superoxide reductases (SOR): rubredoxin-SOR and desulfoferrodoxin (dfx; Erkel *et al*., 2006), however, these two genes were not found in the genome of *Methanosaetaceae concilii* GP-6. Nevertheless, superoxide anions radicals can also be detoxified via a dismutation reaction by superoxide dismutases (SOD; Keith and Valvana, 2007). Several classes of SODs exist which can be distinguished by their metal cofactors and cellular locations. SodC is a periplasmic enzyme containing Cu and Zn as cofactors and is present in *Methanocella arvoryzae* MRE50 (Figure 6.1a). SodA and SodB are cytoplasmic SODs containing Mn and Fe as cofactors (Keith and Valvana, 2007). Interestingly, *Methanosaetaceae concilii* GP-6 encodes for a cytoplasmic iron/manganese superoxide dismutase potentially able to detoxify superoxide anions radicals in the cytoplasma and so compensating the missing cytoplasmic superoxide reductases encoded by *sor* and *dfx* (Figure 6.1b). Until today

three complete genomes of *Methanosaetaceae* are sequenced: *Methanosaeta thermophila* PT (NC_008553.1), *Methanosaeta harundinacea* 6Ac (NC_017527.1) and *Methanosaeta concilii* GP-6 and their genes encoding for oxygen detoxifying enzymes are summarized in Table 6.1.

In summary, the results of the present study indicate that methanogens are strongly susceptible to periods of drought in neotropical forest canopies but may have a higher oxygen tolerance than previously assumed supporting the observations that aerated soils can be inhabited by methanogens (Angel *et al*., 2011; Peters & Conrad, 1995).

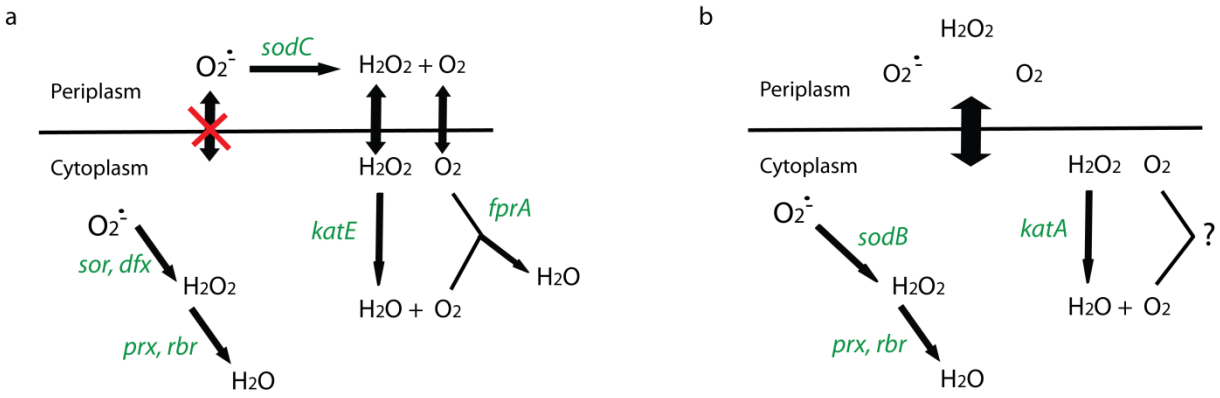

Figure 6.1 Schematic presentation of the predicted enzymatic reactions mediating oxidative stress defense in (a) *Methanocella arvoryzae* MRE50 (formerly known as RC-I$_{MRE50}$, adopted from Erkel *et al*., 2006) and present genes (in green) encoding antioxidant enzymes in (b) *Methanosaeta concilii* GP6 (NCBI reference number: NC_015416.1) Gene abbreviation: superoxide dismutase (*sod*), superoxide-reductase (*sor*), desulfoferredoxin (*dfx*), peroxiredoxin (*prx*), rubrerythrin (*rbr*), catalase (*kat*), $F_{420}H_2$ oxidase (*fprA*).

Table 6.1 Genes of antioxidant enzymes in the genomes of *Methanosaeta concilii* GP-6, *Methanosaeta harundinacea* 6Ac and *Methanosaeta thermophila* PT.

Enzyme (gene)	***Methanosaeta concilii* GP-6**	***Methanosaeta harundinacea* 6Ac**	***Methanosaeta thermophila* PT**
Catalase (*kat*)	+(*katA*)	+	-
Peroxiredoxin/thioredoxin peroxidase (*prx*)	+	+ (putative)	-
Rubrerythrin/rubredoxin peroxidase (*rbr*)	+	+	+
Superoxide dismutase (*sod*)	+(Mn/Fe)	+(Mn/Fe)	+
Superoxide reductase/rubredoxin-superoxide reductase (*sor*)	-	-	-
Desulfoferrodoxin/rubredoxin oxidoreductase (*dfx*)	-	*dfx* domain protein	-
$F_{420}H_2$- oxidase (*fprA*)	-	-	-
Thioredoxin (*trX*)	+	+	+
Rubredoxin (*rub*)	+	+	Rubredoxin-type protein

+, present; -, not present; KatA= type I monofunctional clade III small subunit $heme_b$ catalase; Mn/Fe-SOD = cytoplasmic mangan/iron containing SOD

Nevertheless, in most environments archaeal methanogenesis takes place only in reduced, anoxic environments, and is highly dependent on the availability of usable substrates provided by several microbial guilds (Le Mer & Roger, 2001). For instance, fermenting bacteria are relevant for methanogenesis since they provide the main methanogenic substrates, i.e. H_2, CO_2 and acetate (Noll *et al.*, 2010). Similar to the archaeal community the bacterial community in tank bromeliad substrate revealed to be highly sensitive to drought, upon which their diversity dramatically decreased (**Chapter 4**). However, regardless of the water amendment or the incubation environment (inside or outside of bromeliads) the genus *Burkholderia* was the most abundant group. We assume that the ubiquity of the *Burkholderia* indicates a resistance to changing water levels, which can regularly occur under natural conditions in tank bromeliads depending on the tank capacity, the influence of evaporation (Zotz and Thomas, 1999), the location of the plant, and the precipitation patterns.

With increasing drought also *nifH*, a marker gene for nitrogen fixation, increased. Some *Burkholderia* strains are able to fix nitrogen (Suárez-Moreno *et al*., 2008) and possess the *nifH* marker gene (Caballero-Mellado *et al*., 2007). Dinitrogen gas-fixing microbes can provide nitrogen in form of ammonium which might be taken up by bromeliad plants. For bromeliads of the species *V.gigantea* it was already shown that they prefer nitrogen in form of ammonium (Inselsbacher *et al*., 2007).

Goffredi et al. (2011b) reported the identification of four *nifH* sequences from the tank substrates of Costa Rican tank bromeliads which we assigned to the next pure culture relative as *Agrobacter* sp*., Halorhodospira* sp., *Paenibacillus* sp. and the methanogen *Candidatus* Methanoregula boonei. Therefore, we checked the presence of nitrogen fixing genes in methanogens of *Methanosaetaceae* which, equally as the *Burkholderia,* increased in our tank slurry upon drought (**Chapter 3**). In the genome of *Methanosaetceae concilli* (Barber *et al*., 2011) we found the genes encoding for the nitrogenase subunits (*nifD, nifK, nifH*). We therefore speculate that N_2-fixing bacteria and/or archaea, presumably members of the *Burkholderia* and/or *Methanosaetaceae* may play a role for the nitrogen supply for tank bromeliads especially under water limited conditions. In general, the detection of *nifH* indicates that tank bromeliads inhabiting microbes are not only involved in carbon cycling (e.g. methanogenesis; Martinson *et al*., 2010; **Chapter 3**, Brandt *et al*., 2014) but also in nitrogen cycling.

6.3 Methane emission from phytotelmata

Of identified global CH_4 sources the role of vegetation is the least well understood, although vegetation may provide up to 22% (ca. 32–143 Tg) of the annual flux of CH_4 to the atmosphere (Carmichaeal *et al*., 2014). Indirect emissions of CH_4 by vegetation comprises so called ‘cryptic wetlands’ including bole depressions and phytotelmata (Martinson *et al*., 2010; Carmichaeal *et al*., 2014). Although small in size the vast numbers of plant cisterns could in combination lead to a high cumulative atmospheric CH_4 flux, emphasizing the significance of these ecosystems for the global CH_4 budget (Yavitt, 2010). For instance, this is the first study showing that beside of tank bromeliads, oil palms leaf axils also provide a potential place for methanogenesis (**Chapter 5**). Within the leaf axils of oil palms organic matter accumulates, which presumably gets wet during precipitation,

possibly creating anoxic microniches and a habitat for methanogens as it was also shown for other phytotelmata (Martinson *et al*., 2010; Carmichaeal *et al*., 2014; Krieger and Kourtev, 2012). In incubation experiments of this organic substrate we found that CH_4 is formed under anoxic or water-logged oxic conditions, accompanied by an increase of the methanogenic marker gene *mcrA.*

The results of this work indicate the need to better resolve the role of phytotelmata in the global CH_4 cycle. At the moment it is difficult to give absolute numbers of CH_4 emission rates, since there is little information on phytotelmata densities. Further, environmental changes, precipitation and drought will affect aerobic or anaerobic biochemical reactions. Martinson *et al*. (2010) give a rough estimation that tank bromeliads emit 1.2 Tg CH_4 y^{-1} which accounts for ca. 0.2% of the global annual CH_4 budget (Carmichaeal *et al*., 2014). Nevertheless, Kotowska and Werner (2013) reported that due to within-day fluctuations these values may be overestimated by 20-30%.

In the present study factors were identified that are influencing the inhabiting microbial communities, CH_4 emission as well as the pathway of CH_4 formation in bromeliad tanks. The combination of field and laboratory data revealed new aspects that should be taken into account when assessing CH_4 emission rates. Together with previous studies the main findings are summarized in table 6.2, which also shows that a combination of ecology and microbiology, field data and laboratory data will advance the knowledge of processes that are involved in CH_4 cycling.

Table 6.2 Summary of factors that influence microbial communities and methane formation in tank bromeliads.

	Influence on CH_4 formation	Influence on microbial community	Key findings	Reference
Tank bromeliad size	Yes	?	• Exponential relationship between CH_4 emission rates and tank diameters	Martinson *et al*., 2010
Plant environment	?	Yes	• Different bacterial community after incubation inside or outside of bromeliad tanks	**Chapter 4**
Desiccation/H_2O availability	Yes	Yes	• Decrease of CH_4 production with increasing drought • Increase of acetoclastic *Methanosaetaceae* with increasing drought • Increase of *Burkholderia* with increasing drought and decrease of bacterial diversity	Kotowska and Werner, 2013 **Chapter 3** **Chapter 4**
pH	?	Yes	• Bacterial and archaeal community is influenced by acid-base conditions	Goffredi *et al*., 2011 **Chapter 2**
Temperature/day-time	Yes	?	• Linear relationship between water temperature and CH_4 efflux	Kotowska and Werner, 2013
Oxygen (O_2)	?	Yes	• Bacterial and methanotrophic community composition affected by O_2 concentration of tank slurry	**Chapter 2**
Carbon (C)	?	Yes	• Bacterial, archaeal and methanogenic community composition affected by C concentration of tank slurry	**Chapter 2**
Nitrogen (N)	Yes	Yes	• CH_4 production limited by N availability • Bacterial and methanogenic community composition affected by N concentration of tank slurry	Kotowska and Werner, 2013 **Chapter 2**
Phosphate (P)	No	?	• Addition of P did not affect CH_4 production	Kotowska and Werner, 2013

Martinson *et al*., 2010: field data/sampling site Ecuador; Kotowska and Werner, 2013 field data & greenhouse experiments/sampling site Ecuador; Goffredi *et al*., 2011: field data/sampling site Costa Rica

6.4 Outlook

The establishment of the tank bromeliad as a model system in the greenhouse facilitated manipulation experiments in a controlled environment. This opens up new possibilities and enables the investigation of plant-microbe interactions.

The results of our pilot study highlight the utility of the model system as a tool to study tank-inhabiting microbial communities and mechanisms, like methanogenesis, under defined conditions (e.g. restricted water availability). We suggest studying the effect of further environmental factors on the microbial community and CH_4 cycling in tank bromeliads. Temperature and substrate quality/quantity are important factors for tank bromeliads and their inhabiting communities. Due to daily and seasonal variations the plant is regularly exposed to temperature shifts which can be easily imitated in the greenhouse. Further, tank bromeliads are solely dependent on the substrate that is fallen into the tank or incooperated by animals for their nutrient demand. Especially nitrogen is a limiting factor for tank bromeliads. Interestingly nitrogen fixing bacteria seem to play a role in tank bromeliads and their presence in tank substrate was confirmed in this thesis (**Chapter 4**). In field studies it was shown that nitrogen has a positive effect on methanogenesis in tank bromeliads (Kotowska and Werner, 2013) but knowledge concerning the microbial processes that are involved is still missing. We therefore propose to investigate the microbial community and the effect on CH_4 production during increased nitrogen availability as well as nitrogen limitation.

Several plants are known to stimulate the microbes in their vicinity by secreting root exudates (Rovira, 1969). For tank bromeliads it is assumed to influence their tank inhabiting microorganisms. A first hint for this is given in **Chapter 4** as the bacterial community differed, when tank substrate was incubated inside or outside of bromeliad tanks. Future approaches should address the interaction of the plant and their inhabiting microbes. For instance, stable isotope tracer studies can reveal if the plant releases exudates into the tank and be used to identify the microbes which take them up.

In general, using the tank bromeliad as model system can help to better understand processes involved in carbon and nitrogen cycling. Then, the gained knowledge has to be taken into account when establishing field experiments. In conclusion, the combination of

in situ gas measurements with advanced molecular techniques enables the identification of plant-microbes interactions in relation to CH_4 emission.

In this thesis, the analysis of single microbial groups in tank bromeliads revealed some interesting aspects. The increased presence of *Methanosaetaceae* under increased oxygen exposure indicates a higher oxygen tolerance than previously assumed. The increasing availability of sequenced genomes can help to identify methanogenic species that encode for oxidant-scavenging genes as it is here shown for *Methanosaeta* spp.. We propose the establishment of oxygen tolerance assays by facing these methanogenic species to increased oxygen concentrations. Using metatranscriptomics and metaproteomics can reveal if the transcription of oxygen detoxifying genes and levels of respective enzymes are upregulated under these conditions. These experiments in combination with CH_4 measurements will more clarify the oxygen tolerance of methanogenic species and how methanogenesis is impacted by increased oxygen exposure.

Beside methanogenic species we were able to prove the presence of aerobic methanotrophs in tank bromeliad slurry (**Chapter 2**). Therefore, we suggest, studying the significance of the CH_4-consuming bacteria in tank bromeliads. In rice paddy soil, methanotrophs attenuate the flux of CH_4 into the atmosphere. There, it was shown that CH_4 oxidation is affected by temperature, moisture and the addition of nitrogen (Cai and Yan, 1999), which is still unknown for tank bromeliads. A further aspect is to reveal if anaerobic CH_4 oxidation is operative in tank bromeliads as it is shown for other aquatic or wetland ecosystems (Deutzmann *et al*., 2014; Hu *et al*., 2014).

Until today, it is solely an assumption that phytotelmata other than tank bromeliads play a significant role in the tropical CH_4 cycle. In **Chapter 5** we gave first evidence that oil palm leaf axils provide a potential habitat for methanogenesis. *In situ* CH_4 measurements at different sites have to be performed to determine the significance of this source in the tropical CH_4 cycle. Simultaneously, a comprehensive sampling of oil palm substrate has to be conducted to identify the active microbial species involved in CH_4 cycling using advanced molecular techniques (e.g. metranscriptomics, metaproteomics).

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Erklärung

Ich versichere, dass ich meine Dissertation

„Methanogenesis in phytotelmata: Microbial communities and methane cycling in bromeliad tanks and leaf axils of oil palms"

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Franziska Brandt

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